Modulation of Sulfate Renal Transport by Alterations in Cell Membrane Fluidity

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Received April 12, 1999. Final revised manuscript received June 28, 1999.
Accepted for publication July 14, 1999.

Abstract □ Changes in membrane fluidity have been shown to alter the sodium-dependent renal transport of glucose and phosphate; however, this has not been examined for sodium/sulfate cotransport in the renal proximal tubule. Sodium/sulfate cotransport regulates the homeostasis of sulfate in mammals. The objective of this study was to investigate the influence of alterations of membrane fluidity on sodium-coupled sulfate transport in the Madin–Darby canine kidney cells, which have been stably transfected with sodium/sulfate cotransporter (NaSi-1) cDNA (MDCK-Si). Preincubation of cells with 0.2 mM cholesterol significantly decreased the V_{max} for sodium/sulfate cotransport (13.69 ± 1.11 vs 10.15 ± 1.17 nmol/mg protein/5 min, mean ± SD, $n = 4$, $p < 0.01$) with no significant alteration in K_m. The addition of benzyl alcohol (20 mM) to cells increased the V_{max} of sulfate uptake by 20% (11.97 ± 0.91 vs 14.35 ± 0.56 nmol/mg protein/5 min, mean ± SD, $n = 3$, $p < 0.05$) with no significant change in K_m. Membrane fluidity, as measured by the fluorescence polarization of 1,6-diphenyl 1,3,5-hexatriene (DPH), was significantly increased in MDCK-Si cells treated with 20 mM benzyl alcohol and decreased in the cells preincubated with 0.2 mM cholesterol, compared with control cells. Our results suggest that alterations in membrane fluidity that may occur as a result of disease states, aging, and pregnancy may play an important role in the modulation of renal sodium/sulfate cotransport.

Introduction

Membrane fluidity (membrane motional order or lipid packing order) affects the activity and kinetics of membrane-bound enzymes and transport carriers, accessibility of membrane receptors, and passive permeability properties of membranes.[1] Membrane fluidity can be determined by two factors: environmental factors, such as temperature, and intrinsic factors, such as membrane composition. Alterations in fluidity have been reported in a variety of physiological/pathological conditions such as aging, organ maturation, ischemia, low phosphate diet, vitamin D-induced hypercalcemia, succinylacetone-induced Fanconi syndrome, streptozotocin-induced diabetes mellitus, and liver disease produced by biliary obstruction.[2−4] Drug treatment including treatment with estrogens, gentamicin, anesthetic alcohols (pentanol, hexanol and heptanol), and salicylic acid can also alter membrane fluidity.[2,5,6]

Cholesterol is an essential constituent of the membranes of mammalian cells and is involved in normal cell growth and function. It is mainly located in the cell plasma membrane and acts as the main lipid rigidifier in natural membranes under physiological conditions.[7] The increase in brush border membrane (BBM) cholesterol content and decrease in BBM fluidity in aged rats are associated with a decrease in the V_{max} of sodium-dependent transport of phosphate in renal BBM.[8] Furthermore, Levi et al.[9] have reported that cholesterol directly modulates renal BBM Na^+/phosphate cotransport activity without affecting Na^+/glucose and Na^+/proline cotransport.

Numerous investigations have used the local anesthetic, benzyl alcohol, to alter the physical properties of membranes by increasing their motional order or fluidity. The increase in renal BBM fluidity produced by benzyl alcohol treatment reduced sodium-dependent glucose transport and stimulated Na^+/phosphate cotranport with no change in the affinities of glucose and phosphate for their transport proteins.[2] Friedlander et al.[10] have also confirmed that the increase in apical membrane fluidity achieved by benzyl alcohol produces an increase in phosphate uptake and a decrease of glucose uptake in renal epithelial cells.

Inorganic sulfate, a physiological anion involved in conjugation reactions involving both endogenous and exogenous substrates, is predominantly reabsorbed in the proximal tubule of the mammalian kidney by a sodium-dependent mechanism at the BBM.[11,12] The sodium/sulfate cotransporter is distinct from sodium-dependent phosphate, amino acid, and glucose transport proteins,[13] and the NaSi-1 cDNA identified by Markovich et al.[14] is involved in renal sodium-dependent sulfate transport at the BBM. Unlike other renal sodium-coupled solute transport systems, the effect of membrane fluidity on sodium/sulfate cotransport has not been studied. The objective of the present investigation was, therefore, to examine whether the alterations in membrane fluidity produced by preincubation with cholesterol or by benzyl alcohol treatment affect the activity of the apically located sodium-dependent sulfate transport protein in Madin−Darby canine kidney cells (MDCK) which have been stably transfected by NaSi-1 cDNA (MDCK-Si).[15]

Materials and Methods

Materials—$^{35}SO_4^{2-}$ (as Na_2SO_4, 1050−1600 Ci/mmol) was obtained from New England Nuclear Research Products (DuPont Company, Boston, MA). Biodegradable counting scintillant was purchased from Amersham Co. (Arlington Heights, IL). Commassie blue dye reagent concentrate and bovine plasma γ-globulin protein standard were supplied from Bio-Rad (Richmond, CA). Dulbecco's modified Eagle's medium, fetal bovine serum, and trypsin were obtained from Gibco BRL (Buffalo, NY). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or J. T. Baker (Phillipsberg, NJ).

Cell Culture Conditions—MDCK cells which had been stably transfected with NaSi-1 cDNA (MDCK-Si)[15] were maintained in Dulbecco's modified Eagle's medium, which contained 22 mM $NaHCO_3$, 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL

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10.1021/js990114c CCC: $18.00
Published on Web 08/25/1999

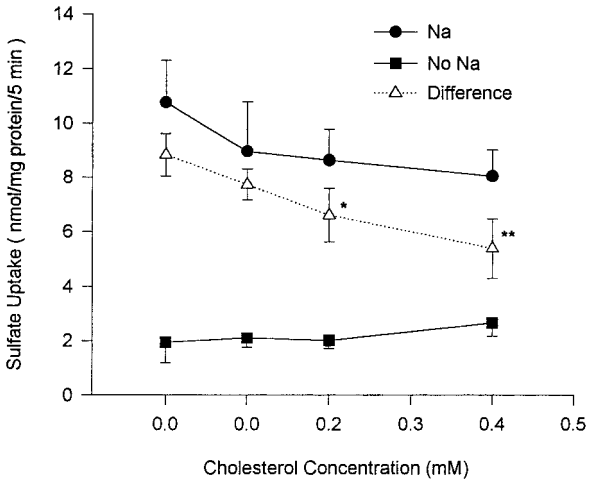

Figure 1—Concentration-dependent effect of cholesterol on sulfate uptake in MDCK-Si cells. Uptake rates were determined at 5 min in the presence of NaCl or of *N*-methyl-D-glucamine. Each data point is the mean ± SD of three separate experiments in which triplicate determinations were obtained. * $p <$ 0.01 compared with control (0 mM cholesterol), ** $p < 0.001$ compared with control.

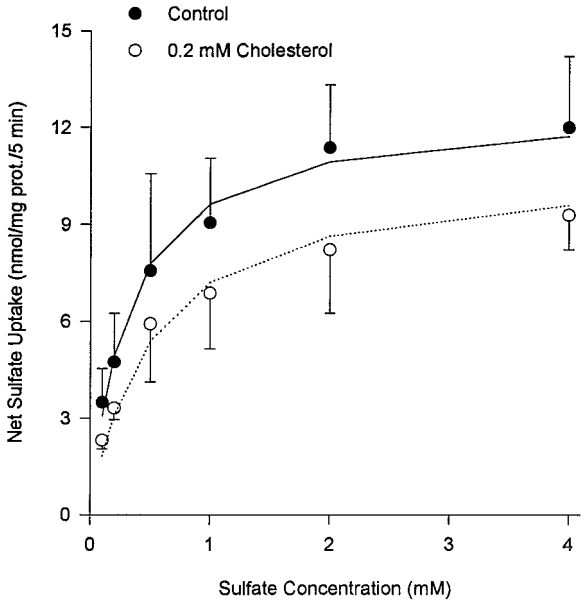

Figure 2—Concentration-dependent sulfate uptake in 0.2 mM cholesterol-preincubated MDCK-Si cells. Sodium-dependent sulfate uptake was calculated as the difference between sulfate uptake rates determined with and without sodium. Each data point is the mean ± SD from four separate preparations, with triplicate determinations of uptake in each preparation. The data were fitted to the Michaelis–Menten equation using nonlinear regression analysis, and the lines represent the fitted lines for the mean data.

streptomycin, 1% nonessential amino acids, and 10% fetal bovine serum, under an atmosphere of 95% air/5% CO_2 at 37 °C. MDCK-Si cells were used up to 15 passages and induced by an incubation with 10^{-6} M dexamethasone for 16 h before sulfate uptake studies.[15]

Sulfate Uptake Studies—The uptake of inorganic sulfate was determined in MDCK-Si cells grown to confluency on culture dishes (35 mm), as described by Hansch et al.[16] Uptake studies were performed at room temperature in a buffered solution which consisted of 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM HEPES/Tris (pH 7.4) containing K_2SO_4 and tracer amounts of radiolabeled sulfate (2 μCi/mL). At the end of incubation, the uptake was stopped by washing the cells three times with ice-cold stop solution (137 mM NaCl, 10 mM Tris/HCl, pH 7.4). Cells were then solubilized with 1% Triton X-100, and radioactivity and protein concentrations of aliquots were determined by liquid scintillation counting and the Coomassie blue binding method[17] using bovine plasma γ-globulin as the protein standard, respectively.

For estimation of transport kinetic parameters, V_{max} and K_m, for sulfate BBM transport, sulfate uptake into MDCK-Si cells was determined at 5 min at various concentrations of sulfate (0.1−6 mM) in the presence and absence of NaCl. For studies done in the absence of sodium, NaCl was replaced by an equimolar amount of *N*-methyl-D-glucamine/HCl. The difference between the uptake rates at the same sulfate concentrations with and without sodium represents the sodium-dependent transport process. Preliminary studies have demonstrated that the 5 min uptake value provides an estimate of the linear uptake of sulfate in MDCK-Si cells. Cells were preincubated with cholesterol at 37 °C for 2 h for the experiments with cholesterol, whereas benzyl alcohol was added to the uptake solution in studies examining the effect of benzyl alcohol on sulfate uptake.

Membrane Fluidity Measurements—The membrane fluidity of intact MDCK-Si cells which were treated with 20 mM benzyl alcohol or preincubated with 0.2 mM cholesterol was examined by measuring the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). MDCK-Si cells were diluted with 2 mL of phosphate-buffered saline solution (PBS, pH 7.4), and 5 μL of 1 mg/mL DPH in tetrahydrofuran was added. One potential problem of measuring fluorescence polarization using the DPH probe in intact cells is the larger particle size of the intact cells which causes depolarization. This problem was partly alleviated by diluting the cells with 2 mL of PBS. Further, the depolarization due to light scattering was corrected by conducting appropriate control experiments as described previously.[18] The binding of the probe to cell components and restricting motions of the probe were evaluated by using various probe-to-cell ratios, produced by serial dilution of the cell suspension.

Fluorescence polarization measurements were performed using a SLM Aminco (SLM Aminco, Urbana, IL) 8000 spectrofluorometer with film polarizers (FP110) at temperatures of 25 °C and 37 °C with the excitation wavelength of 355 nm and the emission wavelength of 430 nm.[6] The temperature equilibration was established by maintaining the chamber at appropriate temperatures for 15 min prior to the measurements using a Neslab (RTE 110) water bath.

Data Analysis—The Michaelis−Menten equation was used to fit the data representing linear sulfate uptake (5 min determinations) over a wide range of concentrations using nonlinear regression analysis (PCNONLIN, Statistical Consultants Inc., Lexington, KY) with a weighting factor, 1/variance, to obtain estimates of the V_{max} and K_m values for sodium/sulfate cotransport.[19] The uptake values were determined in triplicate in any one experiment, and the data from each experiment were fitted to the Michaelis−Menten equation. Three or four experiments were performed, and the mean ± SD for these parameter estimates are reported.

The measured polarization and anisotropy values were used to calculate the lipid order parameter S, using the formula $S^2 = [(4r/3) - 0.1]/r_0$ where r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion (0.40), and r is the steady state anisotropy value. The lipid order parameter was used to estimate the membrane fluidity, as they are inversely related. The static and dynamic components of S were analyzed as described by Pottel et al.[20]

Statistical Analysis—All results were reported as the mean ± SD, unless stated otherwise. The data were compared by unpaired Student's *t*-test between two groups and by ANOVA followed by a Tukey's test among more than two groups. The differences were considered to be statistically significant when $p < 0.05$.

Results

Time Course of Sulfate Uptake—Sodium-dependent sulfate uptake into MDCK-Si cells was examined at various times and increased linearly with time, up to 20 min of incubation. The uptake of sulfate in cells reached a plateau after 60 min, indicating equilibrium (data not shown).

Effect of Cholesterol on Sulfate Uptake—In all studies, sulfate uptake in MDCK-Si cells was significantly increased in the presence of sodium compared with that in the absence of sodium, indicating the sodium dependence

Table 1—Transport Parameter Estimates for Sodium-Dependent Sulfate Uptake into Cholesterol-Preincubated MDCK-Si Cells[a]

	V_{max} (nmol/mg prot/5 min)	K_m (μM)
control	13.69 ± 1.11	435.5 ± 142.8
0.2 mM cholesterol	10.15 ± 1.17[b]	416.7 ± 153.9

[a] Values are presented as mean ± SD of four separate experiments. The estimate for V_{max} was significantly different in control and cholesterol-preincubated cells ($p < 0.01$) with no significant change in the K_m value. [b] $p < 0.01$.

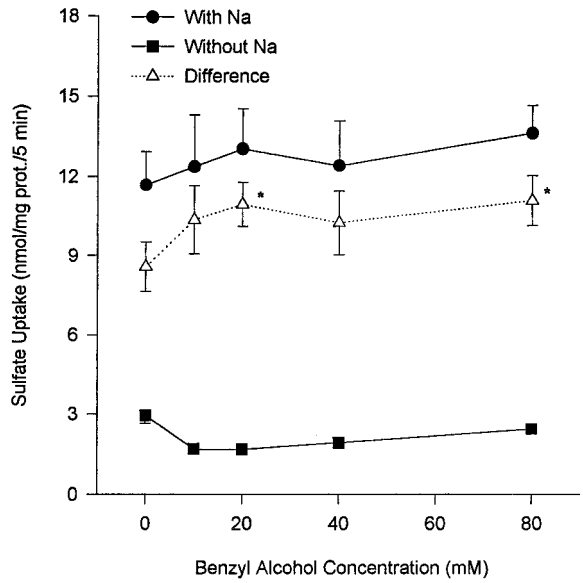

Figure 3—Concentration-dependent effect of benzyl alcohol on sulfate uptake in MDCK-Si cells. Sulfate uptake rates at 5 min were examined in the presence and absence of sodium. Each data point is the mean ± SD of four separate preparations in which triplicate determinations were obtained. * $p < 0.05$ compared with control (0 mM benzyl alcohol).

Table 2—Transport Parameter Estimates for Sodium-Dependent Sulfate Uptake into Benzyl Alcohol-Treated MDCK-Si Cells[a]

	V_{max} (nmol/mg prot./5 min)	K_m (μM)
control	11.97 ± 0.91	464.1 ± 55.9
20 mM benzyl alcohol	14.35 ± 0.56[b]	406.9 ± 174.7

[a] Values are reported as mean ± SD of three separate experiments. The V_{max} value was statistically different in control and benzyl alcohol-treated cells ($p < 0.05$) with no significant difference in the K_m estimate. [b] $p < 0.05$.

of the transport process (Figure 1). Increasing concentrations of cholesterol reduced sulfate uptake in a dose-dependent manner with no significant influence on the sodium-independent component of sulfate transport (Figure 1). The concentration-dependent sulfate uptake in MDCK-Si cells preincubated with 0.2 mM cholesterol is presented in Figure 2. Over a wide range of sulfate concentrations, sodium-dependent sulfate uptake in cholesterol-preincubated cells was decreased compared with the control group. The V_{max} estimate for sodium/sulfate cotransport in cholesterol-enriched cells was significantly lower than that in control cells, while the K_m value was not significantly altered (Table 1).

Effect of Benzyl Alcohol on Sulfate Uptake—Benzyl alcohol increased sodium/sulfate cotransport activity in MDCK-Si cells (Figure 3). As shown in Table 2, the increase of sodium-dependent sulfate uptake in the presence of 20 mM benzyl alcohol (Figure 4) resulted from a increase in the V_{max} estimate of this transport process with no significant change in the K_m value.

Membrane Fluidity—The steady-state fluorescence polarization of DPH represents membrane motional order

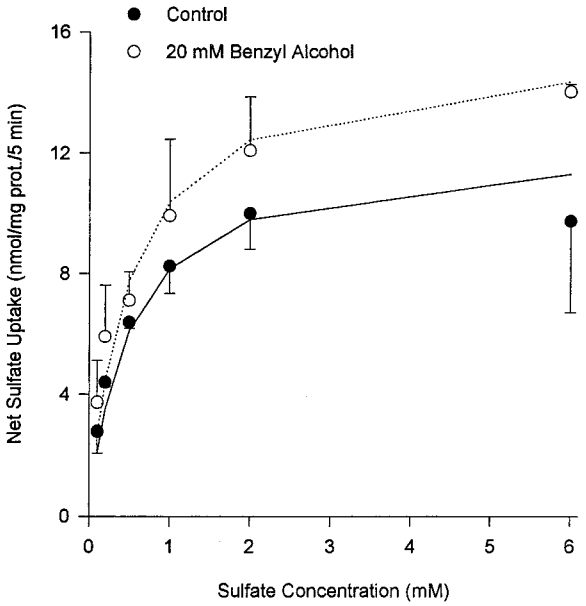

Figure 4—Concentration-dependent sulfate uptake in 20 mM benzyl alcohol-treated MDCK-Si cells. Sodium-dependent sulfate uptake was determined from the difference between uptake rates at 5 min measured in the presence of NaCl or of *N*-methyl-D-glucamine. Each data point is the mean ± SD from three separate experiments, with triplicate determinations of uptake in each preparation. The data were fitted to the Michaelis–Menten equation using nonlinear regression analysis, and the lines represent the fitted lines for the mean data.

Table 3—Lipid Order Parameter and Fluorescence Polarization of DPH in MDCK-Si Cells[a]

temperature	sample	polarization	anisotropy	lipid order parameter (*S*)
25 °C	control	0.396 ± 0.008	0.3041	0.8738
	benzyl alcohol	0.351 ± 0.002[b]	0.2650	0.7958
	cholesterol	0.419 ± 0.009[b]	0.3246	0.9121
37 °C	control	0.292 ± 0.014	0.2156	0.6845
	benzyl alcohol	0.212 ± 0.010[b]	0.1520	0.5066
	cholesterol	0.365 ± 0.011[b]	0.2770	0.8205

[a] Values for fluorescence polarization are expressed as mean ± SD from five measurements. The fluorescence polarization of DPH as a function of temperature was determined in MDCK-Si cells treated with 20 mM benzyl alcohol and preincubated with 0.2 mM cholesterol as well as in control cells. Benzyl alcohol treatment significantly decreases and cholesterol treatment significantly increases the fluorescence polarization of DPH in intact MDCK-Si cells at both temperatures, compared with the value in control cells. The data were compared by ANOVA followed by a Tukey's test among three groups at same temperature. [b] $p < 0.001$.

in the hydrophobic core of the membrane lipid bilayer and is inversely related to membrane fluidity.[6] The calculated lipid order parameter *S* is a measure of lipid packing of the bilayer and can be defined as fluidity. The observed changes in *S* indicate that benzyl alcohol treatment resulted in the fluidization of the membrane, and cholesterol treatment produced a more rigid membrane. The membrane fluidity of MDCK-Si cells treated with 20 mM benzyl alcohol was significantly higher than that of control cells at 25 °C and 37 °C. Cholesterol, at a concentration of 0.2 mM, significantly reduced the membrane fluidity of the cells at both temperatures, compared with controls (Table 3).

Discussion

Changes in membrane fluidity and/or membrane composition have been reported in various physiological and

pathological conditions. Devi et al.[21] have reported an age-dependent increase in membrane cholesterol content and a gradual decrease in membrane fluidity during the prenatal period of liver development in humans. Schwarz et al.[22] have also shown that there are age-dependent increases in total cholesterol and the cholesterol/phospholipid molar ratio, as well as a significant reduction in fluidity in basolateral membranes from rabbit proximal colon throughout postnatal maturation. In rat liver plasma membrane, the cholesterol content increases and the extent of unsaturated fatty acids as well as membrane fluidity decrease during aging.[23] In diabetic rats, the fluidity of renal BBM and BLM is reduced due to changes in the composition of fatty acids esterified in membrane phospholipids.[3] Reversible ischemia increases renal BBM fluidity while a decrease in renal BBM fluidity has been observed in hypercalcemia[25] and liver disease.[4]

Previously, we reported that sodium-dependent sulfate transport in renal BBM was increased in young and pregnant guinea pigs.[24] We also observed that the fluorescence polarization of DPH was decreased in BBM isolated from the kidney cortex of young and pregnant animals, indicating an increased fluidity in the hydrophobic core of the membrane. These results suggested that the increase in BBM fluidity may represent one possible mechanism for the observed changes in sodium/sulfate cotransport during development and pregnancy. The purpose of this study was to investigate whether the modulations of apical membrane fluidity affect sodium/sulfate cotransport in MDCK-Si cells. The present investigation represents the first examination of the effects of membrane fluidity on sodium-dependent sulfate transport.

Preincubation of MDCK-Si cells with 0.2 mM cholesterol significantly decreased the membrane fluidity of the cells. This is consistent with the decreased membrane fluidity in the presence of an increased membrane cholesterol content reported in a number of cell lines including akata cells (lymphoid cell line), human umbilical vein endothelial cells, rat aortic smooth cells, guinea pig keratinocytes, human renal carcinoma cells, and rat prostatic epithelial cells.[7,25–30] In the present studies, the increase in cholesterol concentration produced a reduction of sodium-dependent sulfate uptake in cells in a concentration-dependent manner due to a reduction in the V_{max} of sodium/sulfate cotransport in MDCK cells transfected with NaSi-1. Levi et al.[8] also reported a decreased V_{max} for sodium/phosphate cotransport with no change in K_m in the BBM isolated from aged rats which exhibit a decrease in BBM fluidity and an increase in BBM cholesterol content. In addition, the adaptive increases in the V_{max} of BBM sodium-dependent phosphate transport and BBM fluidity as a result of a low phosphate diet were completely reversed by the increase of cholesterol content in BBM.[9] In vitro enrichment of renal BBM with cholesterol decreases sodium/phosphate transport but has no effect on Na^+/H^+ exchange, sodium/glucose, or sodium/proline cotransport activities which suggests the presence of different lipidic environments (microdomains) for different transport carriers and the absence of an effect on the sodium gradient.[2,9]

Addition of 20 mM benzyl alcohol to the uptake buffer significantly increased the membrane fluidity of the MDCK-Si cells at 25 °C and 37 °C in the present investigation. Friedlander et al.[31] have reported that treatment of MDCK cells with increasing concentrations of benzyl alcohol decreases the steady-state anisotropy of propionyl-diphenylhexatriene and trimethylammonium-diphenylhexatriene, which are located in the hydrophilic surface of the lipid bilayer, suggesting an increased cell plasma membrane fluidity. Our results demonstrated a significantly increased sodium-dependent sulfate uptake in MDCK-Si cells in the presence of 20 mM and 80 mM concentrations of benzyl alcohol. The treatment of cells with 20 mM benzyl alcohol produced an increase in the V_{max} of sodium/sulfate cotransport activity with no change in K_m value. We have also found that benzyl alcohol, at 5 mM and 20 mM concentrations, significantly stimulates the sodium-dependent sulfate uptake at 10 s (linear uptake) in BBM vesicles isolated from the kidney cortex of rats (M. E. Morris, unpublished data). These results are consistent with our previous finding that the increase in sodium/sulfate BBM cotransport was associated with an increased BBM fluidity in young and pregnant guinea pigs.[24] Unlike the effect of benzyl alcohol on sodium/sulfate cotransport, the increase in membrane fluidity by benzyl alcohol altered sodium-dependent phosphate uptake in renal epithelial cells in a bimodal manner: a moderate increase in fluidity (benzyl alcohol concentration less than 20 mM) enhanced phosphate uptake while larger increases reduced it markedly. This biphasic phenomenon for modulation in membrane fluidity by local anesthetics or aliphatic alcohols was also shown for the activity of Na^+,K^+-ATPase,[32,33] whereas fluidization of isolated renal BBM[34] and intact LLC-PK_1 cells[10] decreased the V_{max} of sodium/glucose cotransport in a concentration-dependent manner. Benzyl alcohol concentrations greater than 30 mM also resulted in an increased affinity for glucose for the transporter in kidney BBM vesicles.[34] Therefore, membrane fluidity may influence not only the V_{max} but also the K_m of the substrate for a transporter. The different alterations in the activities of the apically located transporters involved in the sodium-dependent uptake of phosphate, glucose, and sulfate, following the same modification of membrane fluidity, suggest that the lipidic domain around each carrier may be different and the sodium-dependent sulfate transport protein is distinct from sodium/phosphate and sodium/glucose cotransporters.[2,13] Additionally, the different alterations observed for these sodium-dependent transport processes suggest that a change in the driving force (sodium gradient) is not responsible for changes in the observed transport.[2]

Based on the fluidity measurements, we speculate that membrane fluidity changes alter the sulfate transport through altered domain dynamics (altered lipid mobility within a domain). Even though DPH may not be a suitable probe to investigate the lamellar domain properties, due to their fluorescence lifetime alterations (thus polarization), DPH can provide qualitative information regarding the dynamics of lamellar domains. In studies carried out using DPH-labeled artificial membranes, the probe showed higher fluorescence polarization values for the gel phase and reduced polarization values as the fraction of liquid crystalline phase increased.[6,35] This is due to the changes in the fluorescence lifetime of the probe as a result of altered molecular motions. The fluorescence lifetime values decrease from 10.5 to 7.5 ns, as the bilayer transfers from a solid gel phase to a more fluid liquid crystalline phase.[35] Thus, polarization values provide qualitative information regarding the domain formation. In the present study we observed that benzyl alcohol-treated cell membranes are more fluid (decrease in polarization values) as a result of an increase in the fraction of liquid crystalline domains. This alteration in the domain size and dynamics may alter the lipid mobility in the lamellar plane in which the protein is embedded. A secondary effect of this change in lipid dynamics is that the percolation of lipids at the protein–lipid interface and in lipid–protein domains may be altered.

In conclusion, the decrease in fluidity of apical membranes of MDCK-Si cells produced by preincubation with cholesterol resulted in a reduction of sodium/sulfate cotrans-

port whereas the increase in apical membrane fluidity of the cells treated with benzyl alcohol produced an increase in sodium-dependent sulfate uptake. These results suggest that membrane fluidity may play an important role in the regulation of sulfate renal reabsorption in physiological/pathological conditions, including aging and pregnancy.

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Acknowledgments

This work was supported by NSF grant IBN 9629470 and grants from the Western New York Kidney Foundation/Upstate Transplant Services and the Kapoor Charitable Foundation at SUNY at Buffalo (for M.E.M.). Additional support was provided by the Swiss National Science Foundation (for H.M.).

JS990114C