Acute and chronic changes in cholesterol modulate Na-Pi cotransport activity in OK cells

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Breusegem, Sophia Y., Nabil Halaihel, Makoto Inoue, Hubert Zajicek, Eleanor Lederer, Nicholas P. Barry, Victor Sorribas, and Moshe Levi. Acute and chronic changes in cholesterol modulate Na-Pi cotransport activity in OK cells. Am J Physiol Renal Fluid Electrolyte Physiol 289: F154–F165, 2005. First published March 15, 2005; doi:10.1152/ajprenal.00331.2004.—We previously showed an inverse correlation between membrane cholesterol content and Na-Pi cotransport activity during the aging process and adaptation to alterations in dietary Pi in the rat (Levi M, Jameson DM, and van der Meer BW. Am J Physiol Renal Fluid Electrolyte Physiol 256: F85–F94, 1989). The purpose of the present study was to determine whether alterations in cholesterol content per se modulate Na-Pi cotransport activity and apical membrane Na-Pi protein expression in opossum kidney (OK) cells. Acute cholesterol depletion achieved with β-methyl cyclodextrin (β-MCD) resulted in a significant increase in Na-Pi cotransport activity accompanied by a moderate increase in apical membrane Na-Pi protein abundance and no alteration of total cellular Na-Pi protein abundance. Conversely, acute cholesterol enrichment achieved with β-MCD/cholesterol resulted in a significant decrease in Na-Pi cotransport activity with a moderate decrease in apical membrane Na-Pi protein abundance and no change of the total cellular Na-Pi protein abundance. In contrast, chronic cholesterol depletion, achieved by growing cells in lipoprotein-deficient serum (LPDS), resulted in parallel and significant increases in Na-Pi cotransport activity and apical membrane and total cellular Na-Pi protein abundance. Cholesterol depletion also resulted in a significant increase in membrane lipid fluidity and alterations in lipid microdomains as determined by laurdan fluorescence spectroscopy and imaging. Chronic cholesterol enrichment, achieved by growing cells in LPDS followed by loading with low-density lipoprotein, resulted in parallel and significant decreases in Na-Pi cotransport activity and apical membrane and total cellular Na-Pi protein abundance. Our results indicate that in OK cells acute and chronic alterations in cholesterol content per se modulate Na-Pi cotransport activity by diverse mechanisms that also include significant interactions of Na-Pi protein with lipid microdomains.

two-photon fluorescence microscopy; filipin; lipid microdomains; laurdan; opossum kidney cells

DISORDERS OF EXTRACELLULAR inorganic phosphate (Pi) concentration and impairments in renal and gastrointestinal Pi reabsorption are common clinical problems. Aging, diabetes mellitus, malignancy, alcoholism, transplantation, acquired immunodeficiency syndrome (AIDS), and several therapeutic drugs are well known to cause or to be associated with hypophosphatemia or hyperphosphatemia, mainly by affecting renal tubular Pi transport. The kidney plays a critical role in the regulation of Pi homeostasis. The evidence to date indicates that regulation of the overall renal tubular Pi transport by dietary, hormonal, or metabolic factors occurs mainly at the level of the proximal tubular apical brush-border membrane (BBM) Na-Pi cotransport system (36, 53). To date, three distinct families of renal Na-Pi cotransporters have been identified: type I, type II, and type III. These Na-Pi cotransporters are expressed in the proximal tubule of humans, rats, mice, rabbits. Experimental data suggest that the type IIa renal apical BBM Na-Pi cotransport system mediates the majority of the renal proximal tubular BBM Na-Pi transport (1, 2, 6, 19, 22, 26, 27, 29, 30, 35, 49, 56, 59).

Studies to date have determined that dietary factors, hormones, metabolic factors, and the developmental and aging process regulate Na-Pi cotransport activity by diverse molecular and cellular mechanisms, including transcriptional control, translational control, and, most importantly, control via acute trafficking (endocytosis or exocytosis) of the type IIa Na-Pi cotransport protein to and from the apical membrane (36, 53). The net result is that with one known notable exception (61) Na-Pi cotransport activity is directly correlated with apical BBM type IIa Na-Pi cotransport protein abundance.

We have demonstrated that alterations in renal lipid composition, including renal cholesterol, sphingomyelin, and glycosphingolipid content, play an important role in the regulation of renal Na-Pi cotransport activity. Specifically, adaptation to changes in dietary Pi, as well as the aging process are associated with alterations in apical BBM cholesterol content, and there is an inverse relationship between BBM Na-Pi transport activity and BBM cholesterol content (25, 34). In addition, in diabetes, in dietary potassium deficiency, and following treatment with glucocorticoids, there is an inverse relationship between BBM Na-Pi transport activity and BBM sphingomyelin and glycosphingolipid (glucosylceramide and ganglioside GM3) content (27, 61). Furthermore, we have shown that inhibition of glucosylceramide and ganglioside GM3 synthesis results in modulation of Na-Pi transport activity (27, 61).

While our previous studies do indicate that alterations in cholesterol and glycosphingolipid composition modulate renal Na-Pi cotransport activity, the mechanisms by which lipids modulate Na-Pi cotransport activity have not been determined and remain unknown.

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The purpose of the present study was to determine the effects of alterations in cell cholesterol content on the regulation of Na-Pi cotransport activity and Na-Pi protein expression in opossum kidney (OK) cells, a cell line that expresses the fully functional type IIa Na-Pi cotransport system (3, 50).

We have found that acute enrichment of OK cell cholesterol content causes a significant decrease in Na-Pi cotransport activity with a moderate increase in apical membrane Na-Pi protein abundance in the absence of any changes in total cellular Na-Pi protein abundance. In contrast, chronic enrichment of OK cell cholesterol content causes parallel and significant decreases in Na-Pi cotransport activity and apical membrane and total cellular Na-Pi protein abundance as well as decreases in apical membrane lipid fluidity and lipid dynamics.

Acute depletion of OK cell cholesterol content causes increases in Na-Pi cotransport activity with a moderate increase in apical membrane Na-Pi protein abundance in the absence of any changes in total cellular Na-Pi protein abundance. In contrast, chronic depletion of OK cell cholesterol content causes parallel and significant increases in Na-Pi cotransport activity and apical membrane and total cellular Na-Pi protein abundance as well as increases in apical membrane lipid fluidity and lipid dynamics. These results indicate that direct alterations in cell cholesterol content per se are an important modulator of Na-Pi cotransport activity and these effects of cholesterol are mediated by translational (chronic changes in cholesterol) as well as posttranslational (acute changes in cholesterol) mechanisms.

MATERIALS AND METHODS

Materials. Filipin III, β-methyl cyclodextrin (β-MCD), β-MCD/cholesterol complex, protease inhibitors and all other chemicals were obtained from Sigma (St. Louis, MO) except when noted. Cell culture media were also from Sigma. Sera and antibiotics were from Invitrogen (Carlsbad, CA), except for lipoprotein-deficient serum (LPDS), which was purchased from Intracel (Rockville, MD).

Cell culture. OK cells, a renal proximal tubular cell line derived from the opossum kidney (4), were grown in a humidified 5% CO₂-95% air atmosphere in DMEM supplemented with 10% fetal calf serum (FCS), 100 IU penicillin G, and 100 μg/ml streptomycin. Cells were grown to confluence and then were rendered quiescent for 24 h by serum deprivation in Ham’s F-12/DMEM (1:1, vol/vol) supplemented with 4 mM l-glutamine, pH 7.3. For transport studies, OK cells were grown in 24-well dishes. For isolation of cell membranes, fluorescence spectroscopy, and lipid composition measurements, the cells were grown in 10-cm-diameter tissue culture dishes. For fluorescence microscopic studies, cells were grown on coverslips.

Acute modulation of OK cell cholesterol content. Confluent and quiescent OK cells grown in Ham’s F-12/DMEM (1:1, vol/vol) supplemented with 4 mM l-glutamine, pH 7.3, were treated with 1) 10 mM β-MCD for 30, 45, and 60 min to cause progressive depletion of cell cholesterol (see RESULTS) or 2) 10 mM β-MCD/cholesterol for 30, 60, and 90 min to cause progressive enrichment of cell cholesterol (see RESULTS) (14).

To determine whether the effects of cholesterol modulation on Na-Pi cotransport activity are reversible, we performed add-back (repletion/depletion) experiments (58): 1) the cells were treated with either vehicle or 10 mM β-MCD for 45 min to deplete cholesterol and then one-half of the cells were treated with 10 mM β-MCD/cholesterol for 45 min to replace cholesterol; and 2) the cells were treated with either vehicle or 10 mM β-MCD/cholesterol for 45 min to enrich with cholesterol and then one-half of the cells were treated with 10 mM β-MCD for 45 min to remove the cholesterol.

Chronic modulation of OK cell cholesterol content. Cells were seeded and incubated in DMEM in the presence of 10% FCS for 24 h. Cells were then either 1) grown in regular DMEM medium in the presence of 10% FCS, penicillin, and streptomycin; 2) grown in DMEM plus 5% LPDS for 2 days to deplete the cells of cholesterol; or 3) grown in DMEM plus 5% LPDS for 6 h, to upregulate their low-density lipoprotein (LDL) receptors, and then grown on DMEM plus 400 μg/ml of human LDL (Calbiochem, San Diego, CA) for 2 days (45).

Measurement of transport activity. Before the uptake experiments, OK cell monolayers were rinsed once at 37°C with transport solution containing (in mM) 137 NaCl, 5.4 KCl, 2.8 CaCl₂, 1.2 MgSO₄, and 14 HEPES, pH 7.4 (3). To measure Na-Pi cotransport activity, the cell monolayers were then incubated with 200 μl of the above transport solution also containing 0.1 mM K₃H₈[³²P]PO₄. After 5 min, which represents the initial rate of linear uptake (3), transport was terminated by aspiration of the uptake solution and washing of the monolayers three times at 4°C. The cells were then scraped and analyzed for 1)
which accounts for protein. Alteration of cell cholesterol content modulates Na-Pi cotransport (slope: 0.8958). *Statistically different from control (P < 0.05).

To determine whether alterations in cholesterol modulate Na gradient-dependent P uptake (active transport) vs. Na-independent P uptake (diffusion), uptake was determined in parallel in the presence of 137 mM choline chloride.

To determine whether alterations in cholesterol modulate the activity of other transporters, we also measured Na gradient-dependent uptake of 1) methyl-α-D-glucopyranoside, 2) L-glutamate, and 3) sulfate, as described above.

To determine whether the effects of cholesterol modulation on Na-Pi cotransport activity was mediated by alterations in the maximal capacity/velocity (Vmax) of the Na-Pi cotransporter or the affinity for Pi (Km), we performed transport kinetic studies, measuring Na-Pi cotransport activity in the presence of 25–800 μM extracellular Pi. An equation containing saturable (Michaelis-Menten or transport) and nonsaturable (diffusion) components (20) was used to calculate the Km, Vmax, and Ka coefficients by iterative, nonlinear regression. The fits were accepted when two consecutive iterations changed the sum of squares by <0.01%. As an indication of the goodness of the fits, the correlation coefficient (r) and degrees of freedom are shown (degrees of freedom state the number of data points minus the number of fitted parameters). The significance of the differences among the fits was calculated with an F-test, using the sum of squares and degrees of freedom from the fits. The significances of the differences between kinetic constants were obtained with a t-test. GraphPad Prism 3.0cx software was used for statistical and kinetic analysis.

OK cell membrane preparation. Cells were rinsed three times with cold Tris-buffered saline (TBS) and scraped into 2 ml isolation buffer (5 mM HEPES-KOH, 4 mM EDTA, 1 mM PMSF, pH 7.4) and resuspended with a 22-gauge needle. The homogenate was centrifuged at 17,000 g for 60 min, and the pellet was resuspended in 400 μl of resuspension buffer (50 mM mannitol, 10 mM HEPES-Tris, pH 7.4). Protein measurement was performed by the Lowry assay (31), and equal amounts of total protein aliquots were set up for further processing (Western blotting, fluorescence spectroscopy, and measurement of lipid composition).

SDS-gel protein electrophoresis and Western blotting for Na-Pi protein. OK cell membrane samples were denatured for 2 min at 95°C in 2% SDS, 10% glycerol, 0.5 mM EDTA, and 95 mM Tris+HCl, pH 6.8. Ten micromgrams of membrane protein/lane were separated on 10% polyacrylamide gels according to the method of Laemmli (20) and electrotransferred onto nitrocellulose membranes (55). After blockage with 5% fat-free milk powder plus 1% Triton X-100 in TBS (20 mM, pH 7.3), Western blotting was performed with antisera against the COOH-terminal amino acid sequence of NaPi-4 at a dilution of 1:5,000 (22) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Western blots were developed using enhanced chemiluminescence (Pierce, Bradford, IL). The signals were quantified by densitometry and expressed as nanomoles Pi per milligram protein per 5 minutes.

32PO4 counts and 2) protein content. Transport activity is expressed as nanomoles Pi per milligram protein per 5 minutes.
fied in a PhosphorImager with chemiluminescence detector and densitometry software (Bio-Rad, Richmond, CA).

Surface membrane protein biotinylation. To determine the abundance of NaPi-4 in the apical membrane of OK cells, we used surface biotinylation (12, 15, 16, 39). OK cells grown in six-well plates were rinsed three times with PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ at 4°C. The cells were then incubated with 0.5 ml biotinylation buffer (10 mM triethanolamine, pH 7.4, 2 mM CaCl₂, 150 mM NaCl) containing 1.5 mg/ml sulfo-NHS-SS-biotin (EZ-Link Sulfo-NHS-SS-Biotin, Pierce) for 1 h at 4°C under horizontal agitation. Cells were washed with cold PBS and then with quenching solution (100 mM glycine in PBS) for 20 min at 4°C. After three additional washes with cold PBS, the cells were lysed in 0.4 ml of a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, and protease inhibitors for 15 min on ice, scraped, and homogenized by repeated pipetting. The lysates were cleared at 14,000 g for 5 min at 4°C, and the protein concentration of the supernatants was adjusted to 1 mg/ml. Biotinylated proteins were precipitated overnight by mixing 300 µl of cell lysate with 75 µl of streptavidin-agarose beads (ImmunoPure Immobilized Streptavidin, Pierce) in constant rotation at 4°C. The beads were collected by 2-min centrifugation at 2,500 g, washed twice in 1 ml buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl), then twice more in 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and once in 10 mM Tris-HCl, pH 7.4. The beads were resuspended in 100 µl of 2 × Laemmli buffer containing 100 mM DTT, incubated at 42°C for 30 min, and centrifuged for 2 min at 2,500 g. Twenty-five microliters of supernatant were subjected to SDS-PAGE and blotted with anti-NaPi-4 antibodies as described above.

Fig. 4. Effect of treatment of OK cells with β-MCD or β-MCD/cholesterol on Na gradient-dependent α-methyl-d-glucopyranoside uptake (α-MDG; A), Na gradient-dependent l-glutamate uptake (B), and Na gradient-dependent sulfate uptake (C). Alteration in cholesterol has no effects on Na-α-MGP or Na-glutamate uptake but does regulate Na-sulfate cotransport activity. *Statistically different from control (P < 0.05).

Fig. 5. Effect of treatment of OK cells with β-MCD (squares) or β-MCD/cholesterol (circles) on Na-Pi transport kinetics (triangles, control conditions). A: Pi uptake was performed in the presence of a Na gradient (filled symbols) or a choline gradient (open symbols) and in the presence of 50–2,000 µM Pi. B: Na-independent Pi uptake was subtracted from the total uptake measured in the presence of 137 mM NaCl. The kinetic analysis indicates that cholesterol modulates the Vₘₐₓ of Na-Pi cotransport, whereas cholesterol does not modulate the affinity of the transporter for Pi (Kₘ) or the diffusion of Pi (Kₐ) (see Tables 1 and 2).
Table 1. Kinetic analyses of Na-Pi transport activity in OK cells under acute modulation of the apical membrane cholesterol content

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$K_d$</th>
<th>DF, r</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl gradient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-MCD</td>
<td>2.79±0.44</td>
<td>0.16±0.03</td>
<td>0.14±0.25</td>
<td>9, 0.9608</td>
</tr>
<tr>
<td>Control</td>
<td>1.73±0.38</td>
<td>0.09±0.03</td>
<td>0.40±0.26</td>
<td>9, 0.9687</td>
</tr>
<tr>
<td>$\beta$-MCD/cholesterol</td>
<td>1.50±0.25</td>
<td>0.12±0.03</td>
<td>0.33±0.16</td>
<td>9, 0.9500</td>
</tr>
<tr>
<td>Choline Cl gradient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-MCD</td>
<td>0.36±0.03</td>
<td>11, 0.9510</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.49±0.03</td>
<td>11, 0.8992</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-MCD/cholesterol</td>
<td>0.40±0.03</td>
<td>11, 0.9524</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. OK, opossum kidney; $\beta$-MCD, $\beta$-methyl cyclodextrin; DF, degree of freedom. The kinetic constants were obtained from the fits in Fig. 5 as detailed in MATERIALS AND METHODS.

Measurement of cholesterol content. Lipids from OK cell membranes were extracted by the method of Bligh and Dyer (7), as we have previously described (24, 25, 27). For the acute cholesterol modulation experiments, free cholesterol was determined enzymatically using Amplex Red (Wako Chemicals, Richmond, VA). Measurement of cholesterol content. Lipids from OK cell membranes were extracted by the method of Bligh and Dyer (7), as we have previously described (24, 25, 27). For the acute cholesterol modulation experiments, free cholesterol was determined enzymatically using Amplex Red (Wako Chemicals, Richmond, VA). Measurement of cholesterol content. Lipids from OK cell membranes were extracted by the method of Bligh and Dyer (7), as we have previously described (24, 25, 27). For the acute cholesterol modulation experiments, free cholesterol was determined enzymatically using Amplex Red (Wako Chemicals, Richmond, VA).

Cholesterol content was determined by injection of an aliquot of the lipid extract into a Hewlett-Packard 530-m 50% phenylmethyl silicone column in a Hewlett-Packard 59890 gas chromatograph with a flame ionization detector, run isothermally at 280°C, with coprostanol serving as an internal standard. Area ratios were completed with a Hewlett-Packard 3392A integrator.

F158 CHOLESTEROL MODULATES NA-Pi COTRANSPORT ACTIVITY

Table 2. Statistical analyses of Na-Pi cotransport in OK cells under acute modulation of the apical membrane cholesterol content

<table>
<thead>
<tr>
<th>Fit</th>
<th>$F$-test, $P$ Value</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. $\beta$-MCD</td>
<td>3.279, 0.0453</td>
<td>0.0485*</td>
<td>0.2800</td>
<td>0.9195</td>
</tr>
<tr>
<td>Control vs. $\beta$-MCD/cholesterol</td>
<td>6.452, 0.0037</td>
<td>0.6700</td>
<td>0.3406</td>
<td>0.6106</td>
</tr>
<tr>
<td>$\beta$-MCD vs. $\beta$-MCD/cholesterol</td>
<td>22.399, &lt;0.0001</td>
<td>0.0305*</td>
<td>0.7750</td>
<td>0.6003</td>
</tr>
</tbody>
</table>

The statistical analyses on the results of the fits shown in Fig. 5 and collected in Table 1 were performed as detailed in MATERIALS AND METHODS, the $F$-test reporting on the significance of the differences in the fits, and the $t$-test reporting on the significance of the differences in the kinetic constants. $*P < 0.05$ by $t$-test.
from the top to the bottom of the gradient and analyzed for 1) total protein by BCA protein assay (Pierce), 2) Western blotting for Na-Pi, 3) cholesterol content using an enzymatic assay (Wako Chemicals), and 4) sphingomyelin content using an enzymatic assay as described (18).

Statistical analysis. The results are expressed as means ± SE. The statistical significance of differences was assessed by one-way ANOVA and the Tukey multiple comparison test, whereby P < 0.05 was considered significant. All experiments were repeated at least three times with similar results.

RESULTS

Acute modulation of OK cell cholesterol content. Incubation of OK cells with 10 mM β-MCD resulted in a time-dependent depletion of cell cholesterol, as determined both by a biochemical assay for free cholesterol (Fig. 1A) and by filipin staining and two-photon confocal microscopy (Fig. 1B). Conversely, incubation of OK cells with 10 mM β-MCD/cholesterol resulted in a progressive enrichment of cell cholesterol (Fig. 1).

Acute modulation of OK cell cholesterol content causes significant changes in Na-Pi cotransport activity. Acute increases in cholesterol content caused progressive and significant decreases in Na-Pi cotransport activity, whereas acute decreases in cholesterol content caused progressive and significant increases in Na-Pi cotransport activity (Fig. 2A). The effect of cholesterol on Pi transport activity was specific for Na gradient-dependent Pi transport activity (active Pi transport), as cholesterol had no effect on choline gradient-dependent Pi transport activity (passive Pi diffusion) (Fig. 2B). Correlation analysis between cell cholesterol and Na-Pi cotransport activity revealed an inverse relationship between cell cholesterol content and Na-Pi cotransport activity, with a slope of −0.006175 ± 0.0005841 and r = 0.8958 (Fig. 2C).

Effects of cholesterol on Na-Pi cotransport activity are reversible. Depletion of cholesterol in cholesterol-enriched cells and repletion of cholesterol in cholesterol-depleted cells showed that the effects of cholesterol on Na-Pi cotransport activity are totally reversible (Fig. 3).

Effects of alteration in cholesterol content on the activity of other transporters. We next determined whether the effects of alterations in cholesterol content are selective and specific for Na-Pi cotransport activity. We found that alterations in cholesterol content had no significant effects on Na-α-methyl-D-glucopyranoside (Fig. 4A) or Na-glutamate (Fig. 4B) cotransport activity. In contrast, alterations in cholesterol content had significant effects on Na-sulfate cotransport activity (Fig. 4C).

Acute modulation of OK cell cholesterol content causes significant changes in $V_{max}$ of Na-Pi cotransport activity. We performed kinetic studies to determine whether the effects of cholesterol on Na-Pi cotransport activity are mediated via changes in $V_{max}$ or $K_m$. As shown in Fig. 5, A and B, and in Table 1, the kinetic analysis indicates that 1) cholesterol modulates $V_{max}$ of Na-Pi cotransport activity, such that a reduction of the cholesterol content with β-MCD is accompanied by a 1.6-fold increase in $V_{max}$; 2) there are no significant changes in the affinity ($K_m$) of the Na-Pi cotransporter for Pi; and 3) there are also no significant changes in the diffusion constant ($K_d$) for Pi. This constant was similar in the absence or presence of Na$^+$ and corresponds to the slope of the diffusion-nonsaturable component, which also includes the unspecific binding or background of $^{32}$P$^-$ during the uptake assay. The significance of the differences among the fits was obtained with an F-test, whereas the differences between kinetic constants were assayed with a t-test (Table 2).

Acute modulation of OK cell cholesterol content causes moderate changes in apical membrane Na-Pi protein abundance. Surface biotinylation studies showed that despite marked changes in the $V_{max}$ of Na-Pi cotransport activity, acute alterations in cholesterol caused only moderate changes in surface (apical membrane) Na-Pi protein abundance, whereas...
there were no changes in total cellular Na-Pi protein abundance (Fig. 6). The changes in Na-Pi protein abundance were significant only when cholesterol-depleted cells are compared with cholesterol-enriched cells. The correlation between the NaPi-4 protein abundance in the apical membrane and P_i transport under acute cholesterol modulation is further shown in Table 3.

Chronic modulation of OK cell cholesterol content causes significant changes in Na-Pi cotransport activity and Na-Pi cotransport protein abundance. We also examined the effects of chronic alterations in cell cholesterol content on Na-Pi cotransport activity. We found that when OK cells are grown in the presence of LPDS, there is a decrease in cell cholesterol content (Fig. 7A) that results in a significant increase in Na-Pi cotransport activity (Fig. 7B). However, when OK cells are grown in the presence of LPDS and then treated with LDL, there is an increase in cell cholesterol content (Fig. 7A) that results in a significant decrease in Na-Pi cotransport activity (Fig. 7B). In contrast to the effects of acute alterations in cell cholesterol content, which result only in moderate changes in apical membrane Na-Pi protein abundance in the absence of changes in total cellular Na-Pi protein abundance, chronic alterations in cell cholesterol content result in significant and marked changes in total cellular Na-Pi protein abundance as determined by Western blotting of total OK cell membranes (Figs. 7C and 8) as well as by surface biotinylation that measures apical membrane Na-Pi protein abundance (Fig. 8). The correlation between the NaPi-4 protein abundance in the OK cell apical membrane and P_i transport under chronic cholesterol modulation is summarized in Table 3.

Effect of OK cell cholesterol modulation on cell lipid dynamics. We determined the effects of cholesterol modulation on OK cell lipid dynamics by measuring the GP value of the fluorescent lipid probe laurdan (see MATERIALS AND METHODS and Fig. 9A). Because of fast laurdan photobleaching by one-photon UV excitation, we used two-photon excitation microscopy to obtain laurdan GP images in OK cells. Cholesterol depletion using LPDS resulted in a marked increase in low-GP domains, as seen in cross sections (x-y imaging) as well as in the apical membrane (x-z imaging) of OK cells (Figs. 9B and 10). Spectroscopic measurements using OK cell membranes similarly revealed that cholesterol depletion resulted in a decrease in GP or an increase in membrane lipid fluidity.
In contrast, cholesterol enrichment achieved by loading the cells grown in LPDS with LDL resulted in a marked increase in domains with high GP values, as seen in cross sections (x-y imaging) as well as in the apical membrane (x-z imaging) of OK cells (Figs. 9B and 10). In agreement, spectroscopic measurements using OK cell membranes showed that cholesterol enrichment resulted in higher GP values, i.e., a decrease in membrane lipid fluidity (results not shown).

OK cell Na-Pi protein is localized in lipid microdomains. In view of marked effects of acute and chronic alterations in cell cholesterol content on Na-Pi cotransport activity and apical membrane Na-Pi protein expression, we next determined whether Na-Pi protein is present in cholesterol-, sphingomyelin-, and glycosphingolipid-enriched membrane microdomains (lipid rafts). Using a biochemical approach, OptiPrep gradient flotation in the absence of detergent, we found that a significant fraction of Na-Pi protein was present in the low-density fractions 1–3 (Fig. 11A), which are highly enriched by cholesterol and sphingomyelin (Fig. 11B). In addition, using fluorescence microscopy in OK cells with EGFP-tagged Na-Pi protein (rat NaPi-2), we found that a significant portion of Na-Pi protein localizes in GM1-enriched membrane microdomains (lipid rafts) (Fig. 12).

Fig. 9. A: schematic illustrating how laurdan fluorescence spectroscopy and/or microscopy can report on the packing density of biological membranes. The black triangles represent water molecules. B: effect of cholesterol modulation on OK cell lipid dynamics as determined by generalized polarization (GP) laurdan. GP values are displayed as pseudocolors, with blue representing lower GP values and red higher GP values. Images are 3-dimensional reconstructions of a confluent cell layer, consisting of up to 30 images taken at 0.5-μm intervals as cross sections. Displayed is the whole range of GP values. Images were obtained after incubation with laurdan and acquired by a 2-photon laser-scanning confocal microscope for (from top to bottom) LPDS-treated (cholesterol-depleted) OK cells, control cells, and LPDS/LDL-treated (cholesterol-enriched) OK cells. Compared with control cells, cholesterol depletion causes a decrease in GP laurdan and cholesterol enrichment causes an increase in GP laurdan. However, in all 3 cholesterol conditions, there are heterogeneity and a distribution of GP values in intracellular membranes as well as on the apical plasma membrane, suggestive of membrane lipid domains in the apical plasma membrane.

Fig. 10. Effect of cholesterol modulation on OK cell apical membrane lipid dynamics as determined by GP laurdan. GP values are displayed as in Fig. 9, but only GP values ≥0.4 are shown for (from top to bottom) LPDS-treated (cholesterol-depleted) OK cells, control OK cells, and LPDS/LDL-treated (cholesterol-enriched) OK cells, to show how cholesterol depletion, compared with control cells, causes a decrease in GP and cholesterol enrichment causes an increase in GP.
DISCUSSION

Previous studies have shown that during adaptation to alterations in dietary Pi intake and the aging process, there is an inverse correlation between apical brush-border membrane cholesterol content and renal proximal tubular Na-Pi cotransport activity (25, 34). Although these studies suggested a potential role for cholesterol in regulating Na-Pi cotransport activity, they did not establish a direct role for cholesterol in regulating Na-Pi cotransport activity. Also, these previous studies did not determine the mechanisms by which cholesterol may modulate Na-Pi cotransport activity.

In agreement with our earlier in vivo findings, the present study confirms that an increase in cell cholesterol content causes a decrease in Na-Pi cotransport activity whereas a decrease in cell cholesterol content causes an increase in Na-Pi cotransport activity (Figs. 2C and 7). The acute effects of alterations in cell cholesterol content on Na-Pi cotransport activity are fully reversible, as demonstrated by the depletion/repletion experiments (Fig. 3). Through transport kinetics measurements (Fig. 5), we determined that the effects of cholesterol on Na-Pi cotransport activity are mediated through alterations in the \( V_{\text{max}} \) of Na-Pi cotransport, independently of changes in the affinity of the Na-Pi cotransporter for Pi, or any changes in the diffusion of Pi (Tables 1 and 2). We also show that the effects of cholesterol are quite specific and selective for Na-Pi cotransport activity, as there are no changes in Na-glucose or Na-amino acid cotransport activities (Fig. 4, A and B). However, alterations in cholesterol do modulate Na-sulfate cotransport activity (Fig. 4C), a result that is in agreement with previous observations in Madin-Darby canine kidney cells transfected with the Na-sulfate cotransporter NaSi-1 (23).

One of the new findings of our study is the fact that both acute (30–60 min) and chronic (24 h or longer) alterations in cell cholesterol content, well within the physiological changes that we have previously reported, modulate Na-Pi cotransport activity (Table 3). However, the mechanisms are potentially quite different, as acute alterations in cholesterol cause only moderate changes in apical membrane Na-Pi protein abundance and no changes in total cellular Na-Pi protein abundance (Fig. 6, Table 3). In contrast, chronic changes in cholesterol are associated with marked alterations in apical membrane as well as total cellular Na-Pi protein abundance (Fig. 8, Table 3).

There are several possible mechanisms by which alterations in cell cholesterol content can modulate Na-Pi cotransport activity independently of major changes in apical membrane Na-Pi cotransport protein abundance. One possible mechanism is modulation of Na-Pi cotransport protein activity at the level of the apical membrane. In support of this mechanism, we have previously shown that in isolated renal proximal tubular BBM in vitro enrichment with cholesterol, similar to the levels achieved in the present study, results in a decrease in Na-Pi cotransport activity (24). The in vitro enrichment with cholesterol was accompanied by changes in lipid dynamics that could have an important regulatory effect on the function of the Na-Pi cotransport protein.

Cholesterol is known to associate with sphingomyelin and glycosphingolipids to form lipid microdomains or lipid rafts, which are thought to be associated with specific proteins while excluding others. The apical membranes of renal cells have a very high cholesterol and sphingomyelin content, and we have recently provided biophysical evidence for the presence of lipid rafts or lipid microdomains in these membranes (9, 10, 38). In this study, using the z-sectioning imaging of GP laurdan in the apical membranes of OK cells, we also show evidence for the presence of lipid microdomains (markedly differing GP values), and these domains are further modulated by alterations in OK cell membrane cholesterol content (Figs. 9B and 10). Increasing OK cell cholesterol content results in an increased fraction of apical membrane domains with higher GP values, which indicates an increased proportion of liquid-ordered do-

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**Fig. 11.** A: OptiPrep gradient flotation of OK cell membranes performed in the absence of detergent pretreatment followed by Western blotting of the 8 fractions (see MATERIALS AND METHODS) for Na-Pi protein indicates that a significant fraction of Na-Pi protein is present in fractions 1–3. Samples were electrophoresed in the absence of reducing agents. B: cholesterol (■) and sphingomyelin (○) analysis of the OK cell membrane fractions separated on a no-detergent OptiPrep gradient shows large enrichment of both cholesterol and sphingomyelin in the low-density fractions 1–3.

**Fig. 12.** Confocal microscopy imaging of OK cells. Left to right: fluorescence of enhanced green fluorescent protein (EGFP)-NaP (green), GM1 labeled with cholera toxin B (ChTx; red), overlay of the two images (yellow pseudocolor corresponds to colocalization of green and red), and a cross-section (z-c) image of the OK cell showing apical membrane colocalization of EGFP-NaP, and GM1.
mains that may slow the lateral diffusion and mobility of the Na-P_{i} cotransport protein within the apical membrane lipid bilayer. In contrast, decreasing OK cell cholesterol content results in an increased fraction of apical membrane domains with lower GP values, which indicates a decreased proportion of liquid-ordered domains that, in turn, may increase the lateral diffusion and mobility of the Na-P_{i} cotransport protein within the apical membrane lipid bilayer.

In support of this possibility, many studies have similarly shown that the diffusion of several proteins can be modulated by lipids and/or their partitioning into lipid rafts (11, 32, 33, 43, 47, 51, 54, 57). Several but not all of these studies utilized the fluorescence recovery after photobleaching imaging technique to measure the mobile fraction and the lateral/translational diffusion of the proteins of interest. For example, one study showed that the lateral diffusion of GFP-labeled caveolin is highly restricted. Treatment of the cells with methyl-β-cyclodextrin to extract cholesterol and cytochalasin D to disrupt the actin cytoskeleton resulted in increased diffusion of the GFP-labeled caveolin (54). Similar results following the disruption of the actin cytoskeleton were obtained with GFP-labeled aquaporin-2 (57). Two studies using high-resolution single-particle tracking showed markedly decreased diffusion of raft-associated proteins compared with nonraft proteins and observation of a marked increase in the diffusion of the raft-associated proteins following lowering of the cell cholesterol content (11, 43).

Lipid microdomains or lipid rafts may also play a role in the regulation of Na-P_{i} cotransport activity and Na-P_{i} protein abundance by modulating the trafficking of the Na-P_{i} protein. Cholesterol has been shown to play an essential role in endocytosis and intracellular sorting (28, 37, 40, 41). For example, cholesterol has been shown to be necessary for the invagination of clathrin-coated pits, and cholesterol depletion has been shown to inhibit clathrin-dependent endocytosis (44, 52). Recent studies have shown that Na-P_{i} protein is internalized from the apical membrane in part by a clathrin-dependent mechanism (56). Therefore, cholesterol depletion may cause a decrease in clathrin-mediated endocytosis of Na-P_{i}, resulting in increased apical membrane abundance of Na-P_{i} protein and increased Na-P_{i} cotransport activity. In contrast, cholesterol enrichment may result in increased endocytosis and therefore a decreased apical membrane level of Na-P_{i} protein. Cholesterol also has been shown to modulate the structure of invaginated caveolae and inhibit caveolae-mediated endocytosis (17, 45, 46). At the present time, however, it is not known whether caveolae mediate Na-P_{i} protein endocytosis.

Cholesterol and cholesterol-enriched microdomains also modulate intracellular trafficking and apical sorting of molecules (28, 37, 40, 41). In addition, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins that play an important role in the targeting of proteins from the trans-Golgi network to the apical membrane, as well as transcytosis of proteins from the basolateral membrane to the apical membrane, have recently been shown to be associated with lipid rafts or cholesterol-enriched lipid microdomains (8, 21). Therefore, in OK cells cholesterol may also modulate the apical membrane expression of Na-P_{i} proteins by altering the apical targeting of Na-P_{i} molecules.

Another mechanism by which alterations in cholesterol-enriched lipid microdomains can regulate Na-P_{i} cotransport activity is through modulating signal transduction. The plasma-membranial caveolae and other lipid microdomains/rafts, which are highly enriched in cholesterol and glycosphingolipids, play an important role in this process (48). Signaling molecules enriched in caveolae include PDGF, EGF, and endothelin receptors, heterotrimeric G proteins, diacylglycerol, ceramide, PKC and IP_{3} receptors, adenylyl cyclase, nitric oxide, and MAP kinase (48). Interestingly, alterations in the activity of most of these signaling processes are known to regulate renal Na-P_{i} transport activity (22, 36, 53).

In summary, we have demonstrated that in OK cells acute and chronic alteration in cholesterol content play a direct and important role in modulating lipid dynamics, lipid microdomains, and Na-P_{i} transport activity. Our data have established a significant inverse correlation between OK cell cholesterol content and Na-P_{i} cotransport activity (Fig. 2C). Acute alteration in cholesterol content modulates Na-P_{i} cotransport activity by modulating the apical membrane expression of Na-P_{i} protein without altering the total cellular Na-P_{i} protein content. This suggests posttranslational regulation via acute trafficking of the Na-P_{i} protein, similar to what we have previously described in acute regulation of Na-P_{i} protein and transport activity with parathyroid hormone and P_{i} adaptation (26, 29, 30). In contrast, chronic alteration in cholesterol content modulates Na-P_{i} cotransport activity by modulating both apical membrane and total cellular Na-P_{i} protein abundance, which indicate translational regulation of the Na-P_{i} cotransport protein. These studies are in agreement with our previous in vivo observations in aging rats and in rats adapted to dietary P_{i}, where there is an inverse relationship between BBM cholesterol content and BBM Na-P_{i} cotransport activity (24, 25) and indicate that alterations in cholesterol per se is an important modulator of Na-P_{i} cotransport activity.

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