Membrane cholesterol extraction decreases Na\(^+\) transport in A6 renal epithelia

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Am J Physiol Cell Physiol 290: C87–C94, 2006. First published August 17, 2005; doi:10.1152/ajpcell.00184.2005.—In this study, we have investigated the dependence of Na\(^+\) transport regulation on membrane cholesterol content in A6 renal epithelia. We continuously monitored short-circuit current (Isc), transepithelial conductance (Gt), and transepithelial capacitance (Ct) to evaluate the effects of cholesterol extraction from the apical and basolateral membranes in steady-state conditions and during activation with hyposmotic shock, oxytocin, and adenosine. Cholesterol extraction was achieved by perfusing the epithelia with methyl-β-cyclodextrin (mβCD) for 1 h. In steady-state conditions, apical membrane cholesterol extraction did not significantly affect the electrophysiological parameters; in contrast, marked reductions were observed during basolateral mβCD treatment. However, apical mβCD application hampered the responses of Isc and Gt to hypotonicity, oxytocin, and adenosine. Analysis of the blocker-induced fluctuation in Isc demonstrated that apical mβCD treatment decreased the epithelial Na\(^+\) channel (ENaC) open probability (Po) in the steady state as well as after activation of Na\(^+\) transport by adenosine, whereas the density of conducting channels was not significantly changed as confirmed by Gt measurements. Na\(^+\) transport activation by hypotonicity was abolished during basolateral mβCD treatment as a result of reduced Na\(^+\)/K\(^+\) pump activity. On the basis of the findings in this study, we conclude that basolateral membrane cholesterol extraction reduces Na\(^+\)/K\(^+\) pump activity, whereas the reduced cholesterol content of the apical membranes affects the activation of Na\(^+\) transport by reducing ENaC Po.

epithelial Na\(^+\) channel; Na\(^+\)/K\(^+\)-ATPase activity; short-circuit current; methyl-β-cyclodextrin; channel open probability

CHOLESTEROL IS A PROMINENT component of mammalian plasma membranes and an important factor in determining membrane functions (26). Within the cell membrane, cholesterol plays an active role in regulating the lipid bilayer dynamics and structure by modulating the packing of phospholipid molecules (18). Recent studies have suggested that cholesterol is involved in the assembly and maintenance of sphingolipid- and cholesterol-rich microdomains, called rafts, which have been proposed to act as platforms that have functional implications in signal transduction, intracellular trafficking of lipids and proteins, and translocation of solutes across the membrane (5, 12, 27). It was proposed that cholesterol modulates the activity of various membrane transporters, such as the Ca\(^{2+}\) channel (3), NaP1 cotransporter from renal cells (31), or Ca\(^{2+}\)-ATPase (21) and Na\(^+\)-K\(^+\)-ATPase in a variety of cells, including erythrocytes, endothelial and renal epithelial cells (17, 21, 32).

To date, little evidence has been produced to support abnormal regulation of epithelial Na\(^+\) channel (ENaC)-mediated Na\(^+\) transport in renal epithelial cells induced by changes in the cholesterol content of the cell membrane bilayer. Acting at the apical membrane, ENaC activity is modulated to fine-tune Na\(^+\) reabsorption in a number of tight epithelia to maintain body salt and fluid balance (24). Data obtained regarding A6 renal epithelia have shown that endogenously expressed Na\(^+\) channels are associated with rafts, both intracellularly and on the cell surface (11). Heterologously expressed ENaC also has been described as being incorporated into rafts in COS-7 and human embryonic kidney HEK-293 cells (23). At the same time, reconstitution of functional amiloride (Ami)-sensitive Na\(^+\) channels obtained from A6 cultured renal epithelial cells into artificial planar lipid bilayer membranes has shown that ENaCs are restricted to detergent-resistant membrane microdomains and that preservation of native protein-lipid interactions is important for the biological activity of extracted channels (25). On the other hand, it has been shown that lipid-modifying agents do not affect Na\(^+\) transport in steady-state conditions (25). A recent study addressed the issue of ENaC regulation by the changes in membrane lipid order induced by temperature or by chemical compounds (2).

In the present study, we have investigated the effects of a reduced membrane cholesterol environment on Na\(^+\) transport in A6 renal epithelia. Using electrophysiological tools [continuous recording of short-circuit current (Isc), transepithelial conductance (Gt), transepithelial capacitance (Ct) and by blocker-induced noise analysis], we have compared ion transport before and after membrane cholesterol extraction on either the apical or the basolateral side in steady-state conditions and in response to three stimuli that involve different mechanisms and pathways of activation: 1) a hypotonic shock activates Na\(^+\) transport in A6 renal epithelia through pathways that depend on an extracellular Ca\(^{2+}\)-sensitive mechanism, presumably a Ca\(^{2+}\)-sensing receptor in the basolateral membrane (15); 2) oxytocin (Oxy) uses the cAMP pathway, which enhances Na\(^+\) transport via ENaC insertion; and 3) adenosine, when applied to the basolateral side, activates Na\(^+\) transport alone, whereas it does not affect transepithelial Cl\(^-\) transport, which is not the case for either of the other activators.

Our results show that cholesterol extraction from the apical membranes affects Na\(^+\) transport activation by reducing Na\(^+\) channel open probability (Po) without affecting ENaC inser-

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tion. In contrast, basolateral membrane cholesterol extraction abolishes both steady-state Na\(^+\) transport and its activation by reducing Na\(^+\)/K\(^+\) pump activity.

**METHODS**

**Cell Culture**

A6 cells (obtained from Dr. J. P. Johnson, University of Pittsburgh, Pittsburgh, PA) were cultured to confluence at 28°C in an atmosphere of humidified air supplemented with 1% CO\(_2\). The cells were fed twice weekly with growth medium consisting of a 1:1 mixture of Leibovitz’s L-15 and Ham’s F-12 media supplemented with 10% FBS (Sigma, St. Louis, MO), 2.6 mM sodium bicarbonate, 3.8 mM l-glutamine, 95 IU/ml penicillin, and 95 mg/ml streptomycin. All experiments were performed at room temperature with cells from passages 86–97. For electrophysiological measurements, cells were allowed to form polarized monolayers on permeable inorganic membranes (0.2-mm pore size, Anopore; Nunc Intermed, Roskilde, Denmark). Electrophysiological and volume measurements were performed on similar monolayers cultured between 23 and 30 days.

**Cholesterol Extraction Procedure**

Membrane cholesterol extraction was accomplished under continuous measurement conditions by perfusing the apical or basolateral side of monolayers for 60 min with the indicated saline solution containing either 10 or 20 mM methyl-\(\beta\)-cyclodextrin (m\(\beta\)CD). Because of its high affinity for sterols compared with other lipids, m\(\beta\)CD has been used extensively in recent years as an effective tool for manipulating the cell membrane cholesterol level, both in vitro and in vivo (6, 11, 22). Distinct Na\(^+\) reabsorption-stimulation procedures (hyposmotic shock and treatment with Oxy and adenosine) were applied and performed in the absence and presence of the m\(\beta\)CD solution.

**Electrophysiological Measurements**

To perform the electrophysiological measurements, membranes supporting the confluent cell layer were mounted in an Ussing-type chamber (7) and short-circuited using a high-speed voltage-clamp technique. The equipment and theoretical background for electrophysiological and impedance measurements have been described extensively elsewhere (29).

**Noise Analysis**

The pulse protocol method of blocker-induced noise analysis was used to determine the effect of apical cholesterol extraction on single-channel Na\(^+\) current (\(i_{\text{Na}}\)), channel \(P_{\text{Na}}\), and total channel density (\(N_{\text{Na}}\)) of the Ami-sensitive Na\(^+\) channel during steady-state and adenosine-activated Na\(^+\) transport. Noise analysis was implemented using a reversible blocker of the apical Na\(^+\) channel, 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC), according to theory and protocols described previously (4). The blocker on (\(k_{\text{on}}\)) and off (\(k_{\text{off}}\)) rate constants were calculated by performing linear regression analysis of the corner frequency values (\(f_c\)) recorded at different CDPC concentrations according to a stepwise increase protocol.

Current noise power density spectra were recorded while switching the apical CDPC concentration between 10 and 40 \(\mu\)M every 5 min. Three pulses were applied in each considered state: control, treatment with m\(\beta\)CD, and response to adenosine. CDPC-induced noise measurements were performed for m\(\beta\)CD-exposed cells during the last 30 min of the 1-h treatment. In adenosine-stimulated cells, the noise measurements were started in the highly activated Na\(^+\) transport conditions 30 min after the initiation of the agonist.

**Cell Volume Measurements**

Cell volume changes were monitored by measuring cell thickness (\(T_s\)) as described previously (28). \(T_s\) is expressed as a percentage relative to the value recorded just before imposing the hyposmotic challenge. Averaged values of \(T_s\) were calculated from the recordings corresponding to the beads that remained attached to the monolayer during the entire experiment. \(N\) represents the number of measured tissues, and \(n\) is the number of beads used to calculate the average.

**Membrane Permeabilization**

Nystatin, at a concentration of 50 IU/ml, was used to permeabilize the apical membranes with the aim of further characterizing the changes induced by basolateral cholesterol extraction on Na\(^+\)/K\(^+\)-ATPase activity (20). Progression of the apical permeabilization was evaluated by impedance measurements while recording \(I_a\) and \(G_T\) to monitor changes in the basolateral membrane transport activity.

**Solutions and Chemicals**

Table 1 summarizes the composition of the solutions used for electrophysiological and volume measurements. In this study, we used isosmotic solution (260 mosmol/kg H\(_2\)O), hyposmotic solution (140 mosmol/kg H\(_2\)O), and a solution of 200 mosmol/kg H\(_2\)O, all pH 8.2. Experiments with solutions at 200 mosmol/kg H\(_2\)O were bilaterally isoosmotic.

To extract cholesterol from the apical and basolateral membranes, we used solutions containing 20 mM methyl-\(\beta\)-cyclodextrin (m\(\beta\)CD) in all experiments, except for those in which we evaluated ENaC kinetics in response to adenosine. For these experiments, cells were treated with 10 mM m\(\beta\)CD to avoid chemical waste and to reduce the cost of the experiments because this protocol does not allow recirculation of the perfusing solutions. m\(\beta\)CD at a concentration of 20 mM substantially contributed to the final osmolality of the working solution. Therefore, the m\(\beta\)CD-containing solutions at different osmolalities were prepared by taking into account this contribution. The osmolality of the control solutions was adjusted with sucrose while maintaining the same Na\(^+\) concentration used in the corresponding m\(\beta\)CD experiments (Table 1). m\(\beta\)CD at 10 mM was simply added to the perfusing solution. During stimulation procedures, the concentrations of Na\(^+\) on the apical and basolateral sides were equal to avoid a gradient for this ion across the epithelium and transepithelial currents through the paracellular pathway.

Oxy and adenosine were added basolaterally at a concentration of 0.1 IU/ml and 1 \(\mu\)M, respectively. Ami (0.1 mM) was added to the apical bath to determine the Ami-insensitive component of \(I_a\). CDPC (stock solution in DMSO) was used at concentrations up to 200 \(\mu\)M. Most substances were purchased from Merck, except Ami, Oxy, and adenosine (Sigma), CDPC (Aldrich), and m\(\beta\)CD (Fluka).

**Table 1. Solution composition for electrophysiological recordings**

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>K(HCO(_3))</th>
<th>CaCl(_2)</th>
<th>Sucrose</th>
<th>m(\beta)CD</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL 260-I</td>
<td>135</td>
<td>2.5</td>
<td>1</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>CTRL 140</td>
<td>50</td>
<td>2.5</td>
<td>1</td>
<td>35</td>
<td>20</td>
</tr>
<tr>
<td>m(\beta)CD 140</td>
<td>50</td>
<td>2.5</td>
<td>1</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>CTRL 200-I</td>
<td>80</td>
<td>2.5</td>
<td>1</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>m(\beta)CD 200-I</td>
<td>80</td>
<td>2.5</td>
<td>1</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>CTRL 200-II</td>
<td>102</td>
<td>2.5</td>
<td>1</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>m(\beta)CD 200-II</td>
<td>102</td>
<td>2.5</td>
<td>1</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>CTRL 260-II</td>
<td>114</td>
<td>2.5</td>
<td>1</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>m(\beta)CD 260</td>
<td>114</td>
<td>2.5</td>
<td>1</td>
<td>39</td>
<td>20</td>
</tr>
</tbody>
</table>

Concentrations are given in mM. Numbers in solution names indicate solution osmolalities in mosmol/kg H\(_2\)O. The pH of the solutions was 8.2. m\(\beta\)CD, methyl-\(\beta\)-cyclodextrin; CTRL, control.
corresponding electrical parameters variation for control and cholesterol-depleted cells in each type of stimulation.

Results were expressed as means ± SE along with the number (N) of epithelia investigated. All comparisons between the control and experimental groups were performed using Student’s t-test, and statistical significance was defined as P < 0.05.

RESULTS

Apical mβCD Treatment Depresses Na⁺ Transport Activation in Response to Hypotonic Shock

Initially, tissues were exposed to a hyposmotic solution (140 mosmol/kg H₂O) on the apical side and to an isosmotic solution (260 mosmol/kg H₂O) on the basolateral side, and they were allowed to stabilize to a steady-state level that was maintained for at least 30 min. Control cells were kept as such for an extra period of 60 min and subsequently were subjected to hyposmotic shock induced by sudden reduction of the basolateral solution osmolality (θb) to 140 mosmol/kg H₂O. In parallel experiments the hyposmotic challenge was preceded by apical treatment with 20 mM mβCD for 60 min. Next, the hyposmotic shock was applied in the presence of apical mβCD for this set of tissues.

As described previously (29), after a fast but transient decrease in Cₜ observed within 30 s of hypotonicity, Cₜ exhibited a slow, biphasic increase, reaching a maximum after 18 min of hypotonicity. Likewise, both Iₛ and Gₜ showed a biphasic, synchronous rise but required ~60 min of hypotonicity to reach a plateau. Figure 1 shows the comparative changes in Iₛ, Gₜ, and Cₜ in control and apical mβCD-treated tissues in response to the hypotonic challenge. The corresponding mean values obtained at the basal level and at the end of the hypotonic period for each case are summarized in Table 2 for stimulation type 1. The rise in Iₛ and Gₜ reflects mainly transepithelial Na⁺ absorption. This was demonstrated by adding 0.1 mM Ami to the apical bath at the end of the hyposmotic shock, causing a sudden drop in Iₛ and Gₜ to close to the starting values.

The presence of mβCD in the apical compartment did not significantly affect the electrical behavior of A6 renal epithelia during steady-state conditions. After 60 min of perfusion with the mβCD solution, Iₛ merely changed from 0.8 ± 0.1 to 1.1 ± 0.2 μA/cm² and Gₜ remained constant at 0.07 ± 0.01 mS/cm², whereas Cₜ barely changed from 0.80 ± 0.01 to 0.81 ± 0.01 μF/cm² (N = 5; P > 0.05). However, apical mβCD treatment significantly impaired the activation of Iₛ and Gₜ in response to hypotonicity (Fig. 1 and Table 2): the Iₛ increase was 40% less (P = 0.001), while Gₜ was 43% less stimulated (P = 0.008), compared with control cells. Interestingly, the increase in Cₜ for cholesterol-depleted cells was not significantly different from the control tissues: 73% ± 7% compared with 66% ± 4%, respectively (P > 0.05). In addition, mβCD treatment activated a transient (~2–3 min) apical conductance during hyposmotic conditions. In mβCD-treated cells, immediately after imposing the hypotonic shock, Iₛ

Table 2. Iₛ, Gₜ, and Cₜ responses for control and apically cholesterol-depleted cells during different Na⁺ transport stimulations

<table>
<thead>
<tr>
<th>Stimulation Type</th>
<th>Control</th>
<th>mβCD</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Iₛ, μA/cm²</td>
<td>Gₜ, mS/cm²</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.8±0.1</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Hyposhock</td>
<td>10.5±0.9</td>
<td>0.14±0.01</td>
</tr>
<tr>
<td>ΔHypotonic – basal</td>
<td>9.7±0.8*</td>
<td>0.07±0.01*</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>17.5±1.7</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>25.0±1.9</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td>ΔOxytocin – basal</td>
<td>7.5±0.5*</td>
<td>0.16±0.01*</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>11.2±0.6</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>Adenosine</td>
<td>20.2±0.2</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>ΔAdenosine – basal</td>
<td>9.0±0.7*</td>
<td>0.08±0.01*</td>
</tr>
</tbody>
</table>

Cells were treated with 20 mM mβCD (stimulation types 1 and 2) and 10 mM mβCD (stimulation type 3), respectively. For mβCD-treated cells, the basal values considered are those obtained after 60 min of treatment (see text). For stimulated conditions Iₛ, Gₜ, and Cₜ were recorded at the time of maximal increase. Means ± SE for N tissues were calculated from experiments according to the protocols described in RESULTS. *P < 0.05 and †P > 0.05 for comparison between corresponding electrical parameters variation for control and cholesterol-depleted cells in each type of stimulation.
increased rapidly by 1.6 ± 0.3 μA/cm², while $G_T$ temporarily increased by 0.09 ± 0.02 mS/cm² from the starting values mentioned above (Fig. 1). This phenomenon, absent after basolateral substitution of Cl⁻ for $SO_4^{2-}$, reflects a transient Cl⁻ secretion.

**Apical mβCD Treatment Depresses the Oxytocin Response**

In this set of experiments, tissues were allowed to stabilize in solutions of slightly reduced osmolality (200 mosmol/kg H₂O) to elevate the basal level of Na⁺ transport. In 200 mosmol/kg H₂O solutions, apical cholesterol extraction did not significantly affect the basal electrical parameters: $I_{sc}$ presented a variation from 15.8 ± 0.7 to 15.6 ± 0.7 μA/cm², whereas $G_T$ changed from 0.19 ± 0.01 to 0.18 ± 0.01 mS/cm² ($N = 4$). $C_T$ increased slightly but not significantly, from 0.72 ± 0.02 to 0.74 ± 0.02 μF/cm² ($P > 0.05$). However, with Oxy at the basolateral side, Na⁺ transport activation for apically mβCD-treated cells was likely depressed as it was during the hypotonic response (Table 2, *stimulation type 2*): 45% inhibition for the $I_{sc}$ increase ($P < 0.001$) and 30% less stimulation of $G_T$ ($P = 0.003$) compared with control cells. It is important to note that the $C_T$ values did not differ significantly between cholesterol-extracted and control tissues ($P > 0.05$).

**Effects of Acute Apical mβCD Treatment on Elevated Levels of Na⁺ Transport**

Thus far, the evidence indicated that apical cholesterol extraction apparently did not affect basal Na⁺ transport in isosmotic or 200 mosmol/kg H₂O medium. It is conceivable that mβCD might exert a significant effect at elevated levels of Na⁺ transport. Therefore, we performed experiments in which we monitored the effect of mβCD after maximal stimulation of Na⁺ transport induced by exposing the epithelia to an hypotonic shock. The mβCD treatment was administered after $I_{sc}$ reached almost its maximal value 40 min after the initiation of the hypotonic challenge. Figure 2 displays similar time courses of $I_{sc}$, $G_T$, and $C_T$ associated with this approach in control and apically mβCD-treated cells for the first 30 min after mβCD application. Next, the control cells stabilized at a plateau level, whereas a slight decrease could be observed in the presence of mβCD. However, statistical analysis did not indicate a significant difference between mβCD-treated cells and control cells for the indicated period. At the end of the cholesterol extraction treatment period, $I_{sc}$ measured 6.2 ± 0.4 μA/cm² compared with the control, measured at 7.0 ± 0.5 μA/cm² ($N = 5$). $G_T$ reached 0.11 ± 0.01 mS/cm² in mβCD-treated cells compared with 0.12 ± 0.01 mS/cm² in control cells, whereas $C_T$ measured 0.95 ± 0.06 μF/cm² in mβCD-treated cells compared with 0.98 ± 0.06 μF/cm² in control cells ($P > 0.05$).

**Effects of Apical Cholesterol Extraction in Response to Adenosine**

The results reported thus far indicate that the presence of mβCD in the apical bath depressed Na⁺ transport without affecting the increase in $C_T$. Because $C_T$ is proportional to the area of the apical membrane, it appeared that the treatment with mβCD did not affect membrane trafficking. Therefore, we intended to monitor the changes of Na⁺ channel density during Na⁺ transport activation and the effect of mβCD on this parameter. If a correlation between membrane area and Na⁺ channel density was also maintained in these experiments, the reduction of Na⁺ transport activation would presumably be caused by effects on the individual channel, i.e., $i_{Na}$ or channel $P_o$.

First, we investigated the effects of 10 mM mβCD on $I_{sc}$, $G_T$, and $C_T$ in steady-state conditions and in response to adenosine. Figure 3 shows the time profiles for $I_{sc}$, $G_T$, and $C_T$ in response to adenosine in control and mβCD-treated cells. Apical mβCD treatment did not affect the basal, steady-state values of the monitored parameters significantly. During the treatment, $I_{sc}$ changed from 11.6 ± 0.9 to 12.9 ± 0.4 μA/cm², $G_T$ changed from 0.15 ± 0.01 to 0.16 ± 0.01 mS/cm², and $C_T$ changed from 0.71 ± 0.01 to 0.73 ± 0.01 μF/cm² ($N = 5$; $P > 0.05$). It is important to note that 10 mM mβCD reduced the activation of Na⁺ transport in response to basolateral adenosine to a level similar to that reported above in association with 20 mM mβCD treatment that was used during hypotonic shock and with Oxy stimulation. The mean values obtained for $I_{sc}$, $G_T$, and $C_T$ at basal levels and in response to adenosine stimulation for control and apically mβCD-perfused cells are listed in Table 2, *stimulation type 3*. Thus, for the mβCD-treated cells, $I_{sc}$ stimulation in response to adenosine was inhibited by 47% ($P = 0.002$) and $G_T$ was inhibited by 50% ($P = 0.002$) compared with control cells. Again, in these experiments, the maximum increase in $C_T$ in response to adenosine was not significantly different for both types of tissues, increasing by ~10% in both cases.

**Noise Analysis Parameters**

Blocker rate coefficients and $i_{Na}$, $P_o$, and $N_T$ in relation to apical cholesterol extraction. Noise analysis was used to determine the contribution of $i_{Na}$, $P_o$, and $N_T$ to the measured $I_{sc}$ in steady-state conditions and in response to adenosine for control and apically cholesterol-extracted cells. Figure 4A shows the linear relationship between $2\pi f_c$ (equal to the chemical rate of the current-modulating process) and the CDPC
whereas

0.01 to 0.28

presence of adenosine from 0.46

that the main effect of lowering membrane cholesterol is

courted with 10 mM mCD in the apical solution (values calculated as an average concentration in the absence (control) and presence of 10 mM mβCD in the apical solution (values calculated as an average of 6 recordings). The $k_{on}$ and $k_{off}$ rate constants for CDPC during control periods were consistent with those previously reported (14): $2\pi f_c = 7.78 \cdot [\text{CDPC}]_{\text{ap}} + 252.9$. However, mβCD reduced the $k_{on}$ rate constant but did not affect the $k_{off}$ rate constant ($P > 0.05$): $2\pi f_c = 1.94 \cdot [\text{CDPC}]_{\text{ap}} + 264.8$. The results for $I_{sc}$, $G_T$, and $P_o$ in control conditions (basal level and response to adenosine stimulation) and after 1-h apical treatment with 10 mM mβCD are presented as bar graphs in Fig. 4B. For control cells, adenosine increased $N_T$ from 102 ± 6 to 217 ± 22 μm$^{-2}$ ($N = 4, P < 0.05$). $P_o$ slightly decreased in the presence of adenosine from 0.46 ± 0.02 to 0.36 ± 0.05, whereas $i_{Na}$ changed during the stimulation merely from 0.32 ± 0.02 to 0.30 ± 0.02 pA ($P > 0.05$). During mβCD treatment, $N_T$ changed from 104 ± 7 to 129 ± 12 μm$^{-2}$ ($N = 4; P > 0.05$), whereas $P_o$ decreased significantly from 0.44 ± 0.01 to 0.28 ± 0.04 ($P < 0.05$) and $i_{Na}$ increased slightly from 0.32 ± 0.01 to 0.37 ± 0.01 pA ($P < 0.05$). Even though $P_o$ decreased after cholesterol extraction, the $I_{sc}$ values did not change significantly during 60-min exposure to mβCD, because of the increase in $N_T$ and $i_{Na}$ in the presence of mβCD, adenosine increased $N_T$ to 239 ± 38 μm$^{-2}$ ($N = 4; P < 0.05$), slightly decreased $P_o$ to 0.21 ± 0.04 ($P > 0.05$), and reduced $i_{Na}$ to 0.33 ± 0.02 pA ($P > 0.05$). On the basis of these values, the calculated $I_{sc}$ in response to adenosine was less elevated after treatment with mβCD compared with control cells as confirmed by the $I_{sc}$ recordings. An important finding is that the $N_T$ increase in response to adenosine for the cholesterol-extracted cells was similar to that of the control cells. This result is in agreement with the $C_T$ measurements, indicating that the main effect of lowering membrane cholesterol is reflected in the ENaC activity ($P_o$) and not in the channel trafficking processes at the apical membrane.

Effects of Basolateral Treatment with mβCD on $I_{sc}$, $G_T$, and $C_T$ in Steady-State Level and in Response to Hyposmotic Shock and on Volume Regulation

After the stabilization period, cells were basolaterally perfused with 20 mM mβCD for 60 min. Next, the hyposmotic challenge was induced by decreasing the osmolality of the basolateral solution to 140 mosmol/kg H2O while mβCD was maintained in the hyposmotic perfusate. Figure 5 shows how lowering cholesterol on the basolateral side affects the time courses of $I_{sc}$, $G_T$, and $C_T$. The typical time course for these parameters in control experiments is shown in Fig. 1. During mβCD treatment, $I_{sc}$ significantly decreased from 1.7 ± 0.3 to 0.7 ± 0.2 μA/cm$^2$ ($N = 4; P < 0.05$). Moreover, the stimu-

Fig. 3. Effects of cholesterol depletion on the activation of Na$^+$ transport by adenosine. Time courses of $I_{sc}$, $G_T$, and $C_T$ at 200 mosmol/kg H2O in basal steady-state conditions and in response to basolateral adenosine. Control experiments (solid lines) are compared with experiments performed in the presence of 10 mM mβCD (dotted lines) to extract cholesterol from the apical side ($N = 5$).

Fig. 4. Noise analysis parameters. A: relationship between corner frequency values (chemical rate of the current-modulating process, $2\pi f_c$) and the 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC) concentration in the absence (Control) and presence of 10 mM mβCD in the apical solution. The slope and intercept of the linear regressions for the control and mβCD-treatment, $I_{sc}$, $G_T$, and $C_T$ in each case, respectively. B: results of noise analysis calculations. $I_{sc}$, $G_T$, $N_T$, and $P_o$ values in control (basal level and response to adenosine stimulation) are compared with the corresponding values obtained after apical treatment of the cells for 1 h with 10 mM mβCD. *$P < 0.05$ vs. parameters inside each type of experiment. #$P < 0.05$ vs. control and cholesterol-depleted cells.
increased the apical Na\(^+\) conductance, and as a consequence, the elevated level of cytosolic Na\(^+\) highly activated the extrusion process across the basolateral membrane by the Na\(^+\)/K\(^+\) pump (19). Apical membrane permeabilization was initiated by perfusing the basolateral sides of the cells with a solution containing 20 mM mβCD for 1 h while nystatin was kept in the apical perfusion solution. Treatment with mβCD induced a small, transient increase in \(I_{sc}\), followed by a continuous decrease, indicating alterations at the level of Na\(^+\)/K\(^+\) pump activity. \(G_T\) presented a continuous, slow increase for the first 40 min of cholesterol extraction, followed by a more sharp increase within the last 20 min of treatment toward values indicating tissue damage.

**DISCUSSION**

In this study, we have analyzed the role of a reduced membrane cholesterol environment in regulating electrogenic Na\(^+\)/K\(^+\) pump activity in A6 renal epithelial cells. We extracted cholesterol from either the apical or the basolateral membrane. Removal of membrane cholesterol was achieved by exposing the cells to mβCD, a water-soluble cyclic carbohydrate with high specificity for sterols. During cholesterol extraction, electrophysiological parameters were monitored continuously un-
under two conditions: 1) stationary conditions, i.e., at different steady-state levels of Na\(^+\) channel activation; and 2) in response to three types of stimulating procedures: hypotonic shock and basolateral application of either Oxy or adenosine. The main findings of our study are that 1) cholesterol extraction of the apical membranes does not affect steady-state levels of Na\(^+\) transport but 2) impairs Na\(^+\) transport activation in response to all stimulating procedures by decreasing ENaC P\(_o\) without affecting channel insertion, and 3) cholesterol extraction of the basolateral membranes strongly affects basal levels of Na\(^+\) transport and 4) blocks Na\(^+\) transport activation by impairing the activity of Na\(^+\)-K\(^+\)-ATPase without disturbing cell volume regulation.

Cholesterol Extraction and Steady-State Levels of Na\(^+\) Transport

Constitutive levels of Na\(^+\) transport apparently are not dependent on the level of cholesterol in the apical membranes. This finding is in agreement with studies in which other researchers have reported that agents known to modify the amount of lipids (cholesterol and sphingolipids) in cell membranes did not affect the Ami-sensitive transepithelial current (25). However, in analyzing the current constituents using noise analysis, we observed a significant decrease in ENaC P\(_o\) after apical m\(_{\beta}CD\) treatment. Our noise analysis data suggested that the same level of macroscopic I\(_{sc}\) was maintained by a rise in channel density. The decrease in P\(_o\) is a possible consequence of the change in protein conformation. A potential cause is the distortion of hydrophobic interactions in the phospholipid bilayer after alteration of the physical properties of the membrane lipid environment. Such a concept is supported by the observation that cholesterol removal leads to an increase in membrane fluidity (10) and to a decrease in lipid order (9). The difference in perpectivity between apical and basolateral membranes to m\(_{\beta}CD\) treatment may be due to a dissimilarity in architecture between both borders. The apical side, when exposed to the outside, is covered by an intricate structure of glycosylated proteins and lipids for protection, whereas the basolateral sides need to be more open yet are more vulnerable.

Apical Cholesterol Extraction Impairs Na\(^+\) Transport Stimulation by Lowering ENaC P\(_o\)

Independent of the pathway that leads to Na\(^+\) transport activation in A6 renal epithelial cells, all stimuli used in this study were less effective after apical cholesterol extraction. In addition, noise analysis data indicated a decrease in ENaC P\(_o\) as the main cause of this impediment. Interestingly, the observed decrease in I\(_{sc}\) and G\(_T\) stimulation was not paralleled by a diminished increase in C\(_T\). The absence of a difference in C\(_T\) changes between control cells and apically cholesterol-extracted cells indicates that regardless of the underlying mechanism of activation, whether it is increased insertion or decreased retrieval of ENaC proteins, the mechanism is independent of the cholesterol level in the apical membranes. This hypothesis is supported by the noise analysis data showing that the increase in N\(_T\) between control and apically cholesterol-depleted cells is in the same range.

The observation of reduced P\(_o\) for the Ami-sensitive channels in the presence of m\(_{\beta}CD\) depends on correct interpretation of the data obtained from the I\(_{sc}\) fluctuation measurements (30). These data show that in the presence of m\(_{\beta}CD\), CDPC is less effective in blocking ENaC. This finding was demonstrated by the decrease in the k\(_{on}\) rate constant of the blocker. Such an effect is most likely caused by a direct interaction between the cholesterol-depleting drug and the blocker. Alternatively, a competition between the two compounds for binding to the channel can be considered. A direct binding between m\(_{\beta}CD\) and ENaC is unlikely to occur. Such a mechanism would lower ENaC activity instantaneously, whereas effects on ENaC behavior in the presence of m\(_{\beta}CD\) become apparent only at least 30 min after perfusion with the cholesterol-extracting drug.

Basolateral Cholesterol Extraction Impairs Na\(^+\) Transport Stimulation by Attenuating Na\(^+\)-K\(^+\)-ATPase Activity

Basolateral cholesterol extraction induces more rapid and dramatic changes in the electrical parameters of the epithelium. Both steady-state and hypotonsometrically activated levels of Na\(^+\) reabsorption were inhibited by basolateral m\(_{\beta}CD\) treatment. Na\(^+\) transport across the epithelium requires the concerted activity of both basolateral K\(^+\) channels and the Na\(^+\)-K\(^+\)-ATPases. To evaluate properly the transport processes that take place at the basolateral border independently of ENaC activity in the apical membrane, we electrically uncoupled the two cell membranes by permeabilizing the apical membrane with nystatin. This approach increased Na\(^+\) delivery to the pump nearby to the saturation level, making the observed I\(_{sc}\) an index of the Na\(^+\)-K\(^+\)-ATPase activity. The observed decrease in I\(_{sc}\) during basolateral m\(_{\beta}CD\) treatment is therefore a reliable indication of the lowered activity of the pump. These results are supported by findings reported in other studies that showed that normal functioning of Na\(^+\)-K\(^+\)-ATPase depends on the membrane cholesterol content in a variety of cell types, including renal cells (32). In addition, effects of basolateral m\(_{\beta}CD\) on basolateral K\(^+\) channels is unlikely to be involved, given the ability of epithelia to perform normal RVD in these conditions, a process that requires the full activity of the basolateral K\(^+\) channels. These two observations support the idea that the presence of m\(_{\beta}CD\) at the basolateral border affects the activity of the pump.

Basolateral cholesterol extraction also impairs the C\(_T\) rise during hypotonicity. The transient rise in C\(_T\) during hypotonicity reflects the changes in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that occur during hypotonic shock (14). It is conceivable that the reduced basolateral membrane cholesterol level affects one of the underlying mechanisms. Recently, it was reported that activation of Na\(^+\) transport by hypotonicity in the A6 renal epithelial cells can occur in the absence of [Ca\(^{2+}\)]\(_i\) changes (15). Therefore, it is likely that the observed C\(_T\) changes are not related to the activation of I\(_{sc}\) during the hypotonic conditions. The impaired C\(_T\) rise in response to hypotonic shock for basolateral m\(_{\beta}CD\)-treated cells resembles the C\(_T\) rise that is observed when Mg\(^{2+}\) is included in the basolateral perfusion solutions (14). Basolateral Mg\(^{2+}\) blocks noncapacitative Ca\(^{2+}\) entry into A6 renal epithelial cells that occurs during osmotic adaptation of the cells (13). A similar behavior was observed in rat basophilic leukemia cells, in which m\(_{\beta}CD\) significantly inhibited Ca\(^{2+}\) influx from the extracellular medium but did not affect Ca\(^{2+}\) release from intracellular stores (16).
In conclusion, on the basis of the findings of the present study, considered in combination, it can be expected that a reduced body cholesterol level induced either by pharmaceuticals that block cholesterol synthesis, such as statins, or by compounds that extract cholesterol from the membranes, such as when cyclodextrins are used as vehicles for pharmaceutical delivery, body salt loss may occur as a consequence of impairment in the activation of Na\(^+\) reabsorption in the distal parts of the nephron by both reducing the number of open ENaC channels in the apical membranes and rendering the Na\(^+\)-K\(^+-\)ATPases in the basolateral membranes less effective. The understanding of the mechanisms by which the lipid membrane environment participates in the regulation of ion transport activation in epithelial cells actively involved in salt reabsorption and body fluid control may have broad implications regarding the elucidation of physiological and pathophysiological aspects of blood pressure regulation.

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