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Membrane Tension Modulates the Effects of Apical Cholesterol on the Renal Epithelial Sodium Channel

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Abstract We used patch-clamp techniques and A6 distal nephron cells as a model to determine how cholesterol regulates the renal epithelial sodium channel (ENaC). We found that luminal methyl-β-cyclodextrin (mβCD, a cholesterol scavenger) did not acutely affect ENaC activity at a previously used concentration of 10 mM but significantly decreased ENaC activity both when the cell membrane was stretched and at a higher concentration of 50 mM. Luminal cholesterol had no effect on ENaC activity at a concentration of 50 μg/ml but significantly increased ENaC activity both when the cell membrane was stretched and at a higher concentration of 200 μg/ml. Confocal microscopy data indicate that membrane tension facilitates both mβCD extraction of cholesterol and A6 cell uptake of exogenous cholesterol. Together with previous findings that cholesterol in the apical membrane is tightly packed with sphingolipids and that stretch can affect lipid distribution, our data suggest that membrane tension modulates the effects of mβCD and cholesterol on ENaC activity, probably by facilitating both extraction and enrichment of apical cholesterol.

Keywords Patch-clamp technique · Epithelial sodium channel · Na^+ transport · Cholesterol · Methyl-β-cyclodextrin · Confocal microscopy · NBD-cholesterol · Renal epithelial cell

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Introduction

The epithelial sodium channel (ENaC) plays an important role in regulating sodium reabsorption across the distal nephron cells. Excess sodium reabsorption due to enhanced ENaC activity can lead to volume-expended hypertension, as seen in Liddle's syndrome (Warnock, 1999, 2001). Many neurotransmitters and hormones alter ENaC activity in epithelial cells. These include such diverse agents as adenosine (Ma & Ling, 1996), adenosine 5′-triphophate (ATP) (Ma et al., 2002a), dopamine (Helms et al., 2006), angiotensin II (Peti-Peterdi, Warnock & Bell, 2002), prostaglandin E_2 (Guan et al., 1998), insulin (Blazer-Yost & Cox, 1988), antidiuretic hormone (ADH) (Faletti et al., 2002) and aldosterone (Eaton et al., 2001). Recent studies suggest that the regulation of ENaC by adenosine, insulin and ADH requires the presence of cholesterol in the cell membrane (West & Blazer-Yost, 2005; Balut et al., 2006). Several lines of evidence from model membranes suggest that cholesterol preferentially interacts with sphingomyelin via hydrogen bonding to form specialized microdomains or lipid rafts (Henderson et al., 2004). However, evidence from studies involving native cell membranes does not support this concept (Edidin, 2003). Despite the controversy about the existence of lipid rafts in cell membranes, the importance of cholesterol in regulating ion channel function has been recently noted and continues to be further explored. Cholesterol modulates the function of γ-aminobutyric acid A ($GABA_A$) channels (Bennett & Simmonds, 1996), volume-regulated anion channels (Levitan et al., 2000), large-conductance Ca^{2+}-activated K^+ (BK) channels (Lam, Shaw & Duszyk, 2004), inward rectifier K^+ channels (Romanenko, Rothblat & Levitan, 2002; Fang et al., 2006), Kv1.3 potassium channels (Hajdu et al., 2003; Pottosin et al., 2007) and L-type Ca^{2+} channels (Bowles

et al., 2004). Since it has been suggested that a fraction of ENaCs are located in lipid rafts (Hill, An, & Johnson, 2002), cholesterol may also directly regulate ENaCs.

Based on the measurement of short-circuit current across the renal epithelial monolayer, recent studies have demonstrated that application of methyl-β-cyclodextrin (mβCD, a cholesterol scavenger) or cholesterol to the basolateral side, respectively, reduced or enhanced basal ENaC current and that application of mβCD or cholesterol to the apical side, however, did not affect basal ENaC current (West et al., 2005; Balut et al., 2006). The failure to regulate ENaC by luminal mβCD or cholesterol is not very surprising because, unlike the basolateral membrane, the outer leaflet of the apical membrane of renal epithelial cells is thought to be tightly packed with sphingolipids (Le, Friedlander & Giocondi, 1988; Remaley et al., 1998). Since apical or raft cholesterol is difficult to extract by cyclodextrin or high-density lipoprotein (HDL) (Remaley et al., 1998; Scheiffele et al., 1999), additional assistance may be required for cyclodextrin or HDL to efficiently extract cholesterol in the apical membrane. Mechanical stress may serve such a role because mechanical perturbation facilitates scavenging of cellular cholesterol by HDL (Yancey et al., 1996). It is well known that cholesterol in the plasma membrane can be reduced by desorption into HDL (Johnson et al., 1991; Phillips et al., 1998). Since a considerable amount of HDL is present in the urine of patients with either glomerular diseases (Hotta et al., 2004) or nephrotic syndrome (Streather et al., 1993), ENaC may be regulated by urinary HDL in such patients as a result of reduced apical cholesterol. Therefore, investigating the mechanism by which ENaC is regulated by apical cholesterol should provide important information for the abnormal sodium reabsorption found in glomerular diseases and nephrotic syndrome.

By analyzing single-channel properties via patch-clamp techniques, the present study demonstrates that membrane tension modulates the effects of mβCD and cholesterol on ENaC activity. Confocal microscopy data suggest that this modulation occurs probably by facilitating extraction and enrichment of cholesterol in the apical membrane of renal epithelial cells.

Materials and Methods

Cell Culture

A6 distal nephron cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in plastic flasks in a modified medium containing seven parts Coon's F-12 medium (Invitrogen, Carlsbad, CA), three parts Leibovitz's L-15 medium (Invitrogen), 103 mM NaCl, 25 mM $NaHCO_3$, 25 U/ml penicillin, 25 U/ml streptomycin, 2 mM L-glutamine, 10% fetal bovine serum (Invitrogen) and 1 μM aldosterone (Sigma, St. Louis, MO) at 26°C and 4% CO_2. Cells were removed from the flasks and plated on polyester membrane attached to Snapwell inserts (Corning Costar, Corning, NY). The cells were cultured on permeable supports for 10-14 days to allow them to be fully polarized before patch-clamp experiments.

Chemicals and Solutions

Most chemicals, including water-soluble cholesterol (dissolved in $\sim$0.7 mM mβCD), were obtained from Sigma. 25-*N*-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino-27-norcholesterol (NBD-cholesterol) was purchased from Avanti Polar Lipids (Birmingham, AL). NaCl solution contained (in mM) 100 NaCl, 3.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at a pH of 7.4. Hypotonic NaCl solution contained (in mM) 50 NaCl, 3.4 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES at a pH of 7.4. All the concentrations shown in this article are the final concentrations.

Patch-Clamp Cell-Attached Recordings

Immediately before use, a Snapwell insert was thoroughly washed with NaCl solution (see "Chemicals and Solutions") and transferred into the patch chamber mounted on the stage of a Nikon (Tokyo, Japan) inverted microscope. Using patch-clamp techniques, the cell-attached configuration was established on the apical membrane of A6 cells with polished micropipettes with tip resistance of $\sim$5 MΩ. Under the above culture conditions, a patch seal (seal resistance >5 GΩ) was usually formed after releasing positive pressure in the patch pipette or after applying a slightly negative pressure (<3 cm H_2O). About 10% of patches requiring a stronger negative pressure to form a seal >5 GΩ were excluded from further analyses. In some experiments, the so-called back-filling approach was employed. The tip of the patch pipette was filled with NaCl solution, while the rear part of the pipette was back-filled with a modified NaCl solution containing either 10 mM mβCD or 50 μg/ml cholesterol. The length of the tip part filled with NaCl solution alone was calibrated with trypan blue to allow a latency of approximately 25 min for mβCD or cholesterol to diffuse down the pipette to the patch membrane. Only the patches formed within 15 min after the patch pipette was backfilled were used for this approach. In some patches, after patch formation, a negative pressure (3 cm H_2O) was continuously applied to the

patch pipette through a syringe and monitored by a water-filled U-tube. In order to minimize the effects of mechanical stretch and stretch-induced ATP release on ENaC activity (Ma et al., 2002a), the first 3-min period after patch formation was not used for data analysis. A 3-min period 18-21 min after back-filling of the patch pipette was used for evaluating control channel activity, while a 4-min period 26-30 min after the filling was used for evaluating the effect of either mβCD or cholesterol on ENaC activity. This means that a 5-min window from 21 to 26 min was not used. According to the calibration with trypan blue, this should eliminate possible errors caused by the variability among different patches of times taken for mβCD or cholesterol to reach the patch membrane. To complement the back-filling approach, the effects of either mβCD or cholesterol on ENaC activity were also examined in A6 cells pretreated with each compound for 30 min. Before patching, mβCD or cholesterol was washed out. Single-channel currents were obtained with zero applied pipette potential (i.e., the electrical driving force was voltage-clamped at the resting membrane potential), filtered at 1 kHz and directly recorded on a computer hard disk. Before digitization with pClamp 9 software (Molecular Devices, Palo Alto, Ca), single-channel records were low pass-filtered at 30 Hz. The total numbers of functional channels (N) in the patch were estimated by observing the number of peaks detected on the current amplitude histograms. As a measure of channel activity, NP_o (number of channels [N] $\times$ open probability [P_o]) was calculated by using at least 3 min of a single-channel record, as we previously described (Ma & Ling, 1996). Subconductive events with open times longer than 100 ms were manually counted. Experiments were conducted at 22–23°C.

Confocal Microscopic Analysis of Cholesterol Extraction and Enrichment

To compare with the results from patch-clamp experiments, a fluorescent cholesterol analog, NBD-cholesterol, was used to test how A6 distal nephron cells respond to cholesterol extraction and enrichment. A6 cells were cultured on polyester membrane attached to Transwell inserts for 10-14 days before the experiments. To determine if membrane tension could facilitate the extraction of cholesterol by mβCD, A6 cells were incubated in culture medium containing 5 μg/ml NBD-cholesterol for 12 h. Such a long period of incubation was used to allow NBD-cholesterol to fully incorporate into the apical membrane of A6 cells. Immediately before use, Transwell inserts were thoroughly washed with NaCl solution. The cells were incubated in NaCl solution as a control, NaCl solution and luminal exposure to 10 mM mβCD for 30 min or hypotonic NaCl solution and luminal exposure to 10 mM mβCD for 30 min. To determine if membrane tension could facilitate A6 cell uptake of exogenous cholesterol, A6 cells were incubated either in NaCl solution as a control or in hypotonic NaCl solution for 7 min. NBD-cholesterol (50 μg/ml) was then added to the apical bath, and the cells were incubated for 3 min more. Finally, the polyester membrane was excised from each insert and mounted on a glass slide. The fluorescence of NBD-cholesterol was examined by a Leica (Heidelberg, Germany) confocal microscope.

Statistical Analysis

Data are reported as mean values ± standard error (SE). Statistical analysis was performed with SigmaPlot and SigmaStat software (Jandel Scientific, Corte Madera, CA). Paired t-tests were used to determine statistical significance between two time periods before and after mβCD or cholesterol diffused down to the patch membrane, using the same patch as a control. Student's t-tests were used to determine statistical significance between controls and groups pretreated with either mβCD or cholesterol. Results were considered significant at $P < 0.05$, as we described previously (Ma & Ling, 1996).

Results

Luminal mβCD Does not Acutely Affect ENaC Activity at a Concentration of 10 mM

By measuring short-circuit current across the epithelial monolayer, it has recently been shown that luminal mβCD (a cholesterol scavenger) does not affect basal ENaC activity (West et al., 2005; Balut et al., 2006). We used patch-clamp techniques to further determine how cholesterol affects ENaC activity at the single-channel level. The cell-attached patches were formed on the apical membrane of A6 distal nephron cells. Because ENaC activity is highly variable from patch to patch, the back-filling approach was employed in order to use the same patch as a control, as we reported previously (Ma et al., 2002a) and described in "Materials and Methods." Figure 1a shows a representative ENaC single-channel current of a 10-min record 20-30 min after the patch pipette was filled with NaCl solutioin alone. Consistent with our previous report (Ma & Ling, 1996), the mean NP_o was not changed at least within the time frame we used for the back-filling approach, which was 0.76 ± 0.14 (early) *vs.* 0.67 ± 0.10 (late) ($n = 6$, $P = 0.15$) (Fig. 1b). To determine whether extraction of endogenous cholesterol modulates ENaC, the pipette was back-filled with NaCl solution containing 10 mM mβCD.

Control channel activity was recorded before mβCD diffused down the pipette to the patch membrane. The data demonstrated that luminal mβCD at a final concentration of 10 mM did not acutely affect ENaC activity and current amplitude under control conditions (Fig. 1c). The mean NP_o was not changed after mβCD diffused down the pipette to the patch membrane, which is 0.43 ± 0.09 (before) *vs.* 0.40 ± 0.09 (after mβCD) (n = 6, P = 0.22) (Fig. 1d). Consistent with previous results from short-circuit current experiments (West et al., 2005; Balut et al., 2006), our data suggest that luminal mβCD does not acutely affect basal ENaC single-channel activity at a concentration of 10 mM.

Luminal mβCD Decreases ENaC Activity and Extracts Membrane Cholesterol when the Cell Membrane Is Stretched

Since the cholesterol in the outer leaflet of the apical membrane of renal epithelial cells is tightly packed with sphingolipids (Le et al., 1988; Remaley et al., 1998), mβCD at a concentration of 10 mM may not be able to extract such tightly packed cholesterol in polarized cells as it does in nonpolarized cells. In fact, it has already been shown that mechanical perturbation facilitates scavenging of cellular cholesterol by HDL (Yancey et al., 1996). Therefore, we hypothesize that membrane tension may facilitate the access of mβCD to apical cholesterol and subsequently regulate ENaC. Indeed, when a negative pressure (3 cm H_2O) was continuously applied to the pipette, luminal mβCD at the same concentration of 10 mM strongly reduced ENaC activity (Fig. 2a). In the presence of negative pressure, mβCD reduced the mean NP_o of ENaC from 0.82 ± 0.15 (before) to 0.10 ± 0.03 (after mβCD) (n = 6, $P < 0.01$) (Fig. 2b). In contrast, negative pressure alone did not affect ENaC activity in A6 cells (Fig. 2c), just as we reported previously (Ma et al., 2002a).

To determine if mechanical stretch could facilitate the extraction of cholesterol by mβCD, confocal microscopy experiments were performed to test whether 10 mM mβCD could efficiently extract NBD-cholesterol incorporated in the apical membrane of A6 cells. The data demonstrated

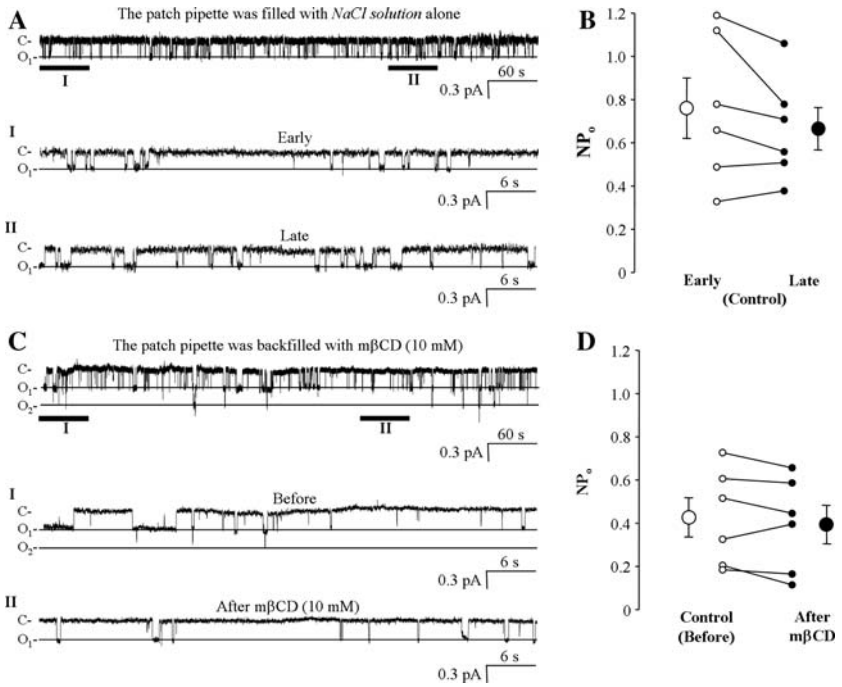

Fig. 1 Luminal mβCD does not affect ENaC under control conditions at a concentration of 10 mM. **a** Representative single-channel current from an A6 cell under control conditions when the patch pipette was filled with NaCl solution alone. Representative single-channel currents in this figure and other figures, unless otherwise indicated, show a 10-min period of ENaC single-channel current 20–30 min after the patch pipette was either filled with NaCl solution alone or back-filled with mβCD or cholesterol. *I* and *II* show zoom-in periods of either early or late ENaC activity, respectively. In all the figures showing single-channel records, downward events represent channel openings; *C-* indicates the baseline when channels are closed; O_1-, O_2-, et al. show open levels. **b** Summary plots of ENaC NP_o values from early (18–21 min after patch pipettes were filled with NaCl solution alone) and late (26–30 min after filling) periods (0.76 ± 0.14 *vs.* 0.67 ± 0.10, n = 6, P = 0.15). **c** Representative single-channel current from an A6 cell before and after mβCD, recorded under control conditions. The patch pipette was back-filled with NaCl solution containing 10 mM mβCD. *I* shows a zoom-in period of control (before) ENaC activity, while *II* shows a zoom-in period of ENaC activity after mβCD diffused down the pipette to the patch membrane. **d** Summary plots of ENaC NP_o values before (18–21 min after the patch pipette was back-filled with 10 mM mβCD) and after (26–30 min after filling) mβCD under control conditions (0.43 ± 0.09 *vs.* 0.40 ± 0.09, n = 6, P = 0.22)

that 10 mM mβCD in the apical bath did not affect cholesterol levels in A6 cells but significantly reduced cholesterol in the apical membrane of cells in the presence of hypotonicity, as shown in Figure 3.

Luminal mβCD at a Higher Concentration Mimics the Effect of 10 mM mβCD Combined with Membrane Tension on ENaC Activity

If the tight packing of the outer leaflet of the apical membrane prevents mβCD extraction of apical cholesterol, a high concentration of mβCD alone may be able to produce a similar effect on ENaC activity. To confirm what we found by using the back-filling approach, A6 cells were pretreated with mβCD at two concentrations (10 and 50 mM) for 30 min. Pretreatment with 50 mM mβCD, but not 10 mM, increased the frequency of subconductive openings (subevents), as shown by a representative single-channel trace (Fig. 4a), from 2 ± 1/min (control, $n = 6$) to 16 ± 3/min (50 mM mβCD, $n = 6$, $P < 0.001$) (Fig. 4b) but decreased the NP_o of ENaC from 1.02 ± 0.21 (control, $n = 8$) to 0.38 ± 0.14 (50 mM mβCD, $n = 8$, $P < 0.05$) (Fig. 4c). These data together with the results from the back-filling approach suggest that either membrane tension or a higher concentration of mβCD is required for luminal mβCD to decrease ENaC activity and facilitate subconductive openings. The requirement of a high concentration of mβCD indicates that cholesterol is tightly packed in the apical membrane of distal nephron cells.

Luminal Cholesterol Increases ENaC Activity and Incorporates into A6 Cell Membrane when the Cell Membrane Is Stretched

The above experiments have shown that membrane tension is required for efficiently extracting endogenous cholesterol to inhibit ENaC activity. If mechanical stretch could modify the tightly packed cholesterol in the outer leaflet of the apical membrane to make it accessible for mβCD, mechanical stretch may also facilitate incorporation of exogenous cholesterol into the tightly packed membrane. To determine the effect of exogenous cholesterol on ENaC activity, the patch pipette was back-filled with NaCl solution containing 50 μg/ml cholesterol. Consistent with previous results from the measurement of amiloride-sensitive current across the renal epithelial monolayer (West et al., 2005), our single-channel recordings showed that luminal cholesterol at a concentration of 50 μg/ml had no effect on ENaC activity under control conditions (Fig. 5a). The mean NP_o was not changed after cholesterol diffused

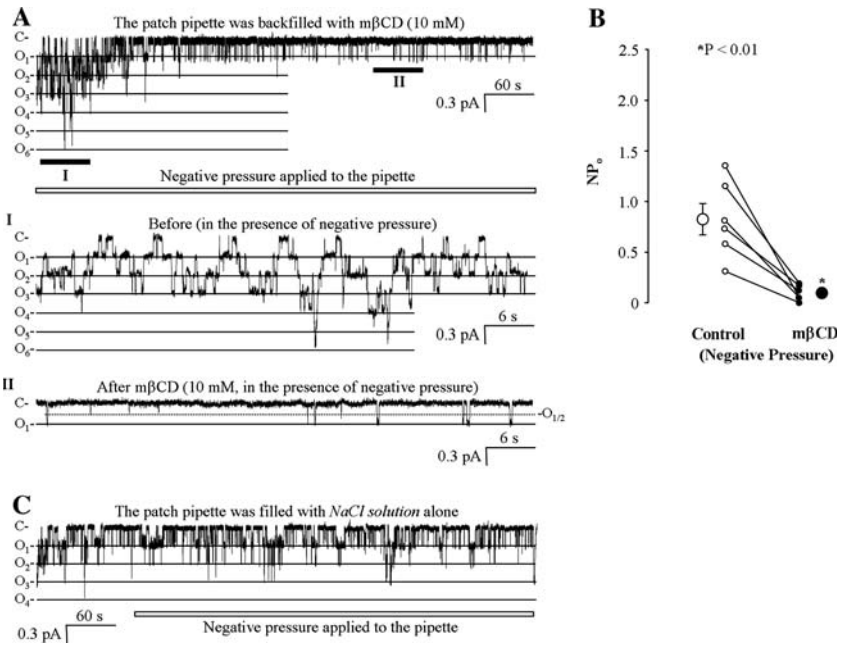

Fig. 2 Luminal mβCD reduces ENaC activity in the presence of a negative pressure. **a** Representative single-channel current before and after mβCD recorded from a cell-attached patch 3 min after negative pressure was continuously applied to the pipette which was back-filled with NaCl solution containing 10 mM mβCD. In this figure and other figures, *-$O_{1/2}$* shows the level when ENaC is at subconductive state. *Narrow open bars* show application of negative pressure (3 cm H_2O) to the patch pipette. **b** Summary plots of NP_o values of unitary conductance before and after mβCD in the presence of negative pressure (0.82 ± 0.15 *vs.* 0.10 ± 0.03, $n = 6$, $P < 0.01$). The same time frames as in Figure 1 were used for calculating NP_o values. **c** Representative single-channel current before and after negative pressure when the patch pipette was filled with NaCl solution alone

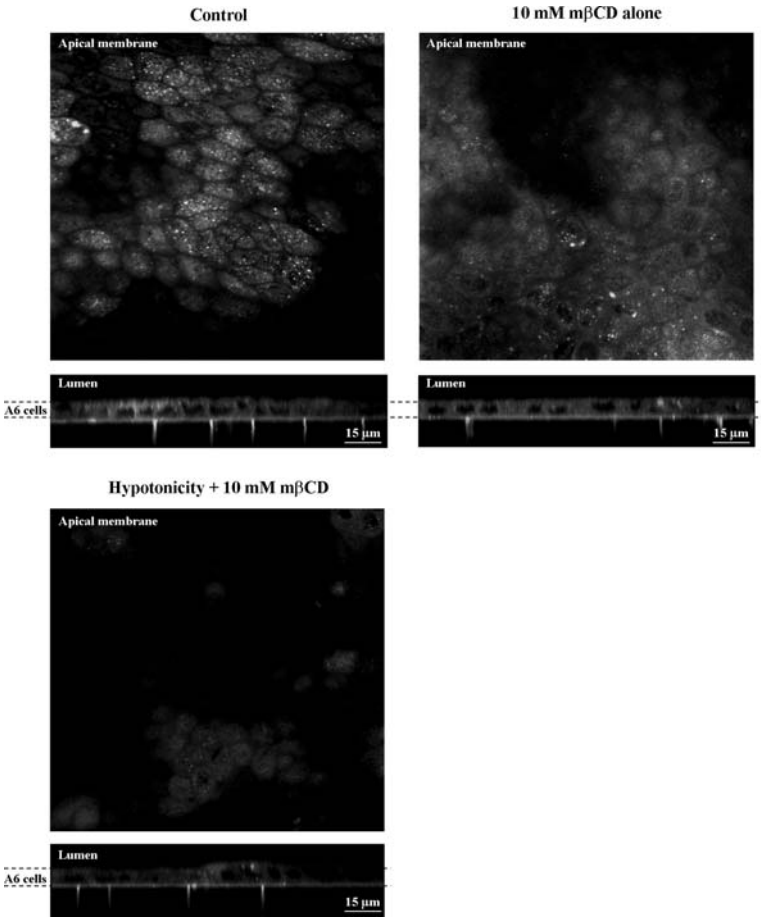

Fig. 3 Membrane tension is required for mβCD extraction of cholesterol in A6 cells. A6 cells cultured on polyester membrane attached to Transwell inserts were incubated in culture medium containing 5 μg/ml NBD-cholesterol for 12 h. The cells were incubated in NaCl solution (*Control, upper left*), NaCl solution and luminal exposure to 10 mM mβCD for 30 min (*10 mM mβCD alone, upper right*) and hypotonic NaCl solution and luminal exposure to 10 mM mβCD for 30 min (*Hypotonicity + 10 mM mβCD, lower left*). *Square images* show confocal microscopy flat view of the apical region of A6 cells, while *rectangular images* show confocal microscopy vertical sections of A6 cell monolayer. In rectangular images, *horizontal fluorescent lines* immediately under the A6 cell monolayer are due to the deposit of NBD-cholesterol on the surface of the polyester membrane, while *vertical fluorescent lines* are due to the deposit of NBD-cholesterol in the pores of polyester membrane. The same gain for detecting the fluorescence was used in each scan. The figure shows representative images from three consistent results

down the pipette to the patch membrane, which is 0.48 ± 0.09 (before) *vs.* 0.49 ± 0.10 (after cholesterol) ($n = 7$, $P = 0.76$) (Fig. 5b). In contrast, luminal cholesterol at the same concentration of 50 μg/ml significantly increased ENaC activity when a negative pressure of 3 cm H_2O was continuously applied to the patch pipette (Fig. 5c). In five of these seven patches, cholesterol appeared to also induce the appearance of significant ENaC subconductance, as shown below (see $O_{1/2}$ in panel II of Fig. 7a). The mean NP_o was increased in the presence of a constant negative pressure, from 0.44 ± 0.12 (before) to 0.68 ± 0.08 (after cholesterol) ($n = 7$, $P < 0.01$) (Fig. 5d).

Also by using NBD-cholesterol, confocal microscopy experiments were performed to determine if mechanical stretch could facilitate A6 cell uptake of exogenous luminal cholesterol. NBD-cholesterol (fluorescence) was not found in the apical membrane of A6 cells incubated in NaCl solution with regular osmolality for amphibian A6 cells (upper left in Fig. 6). Only a horizontal straight line was observed in the image from a vertical section of the A6 cell monolayer cultured on polyester membrane (lower left in Fig. 6). By sectioning the cells, we know that this weak fluorescent line is located under the cells on the surface of the support material, indicating that a relatively low dose of NBD-cholesterol did successfully enter A6 cells but was completely pumped out, probably by cholesterol transporters in the basolateral membrane, and deposited on the surface of support material. In contrast, when A6 cells were incubated in hypotonic NaCl solution with 50% of regular osmolality, a significant amount of NBD-cholesterol was found in the apical membrane (upper right in Fig. 6), even though a portion of NBD-cholesterol was pumped out,

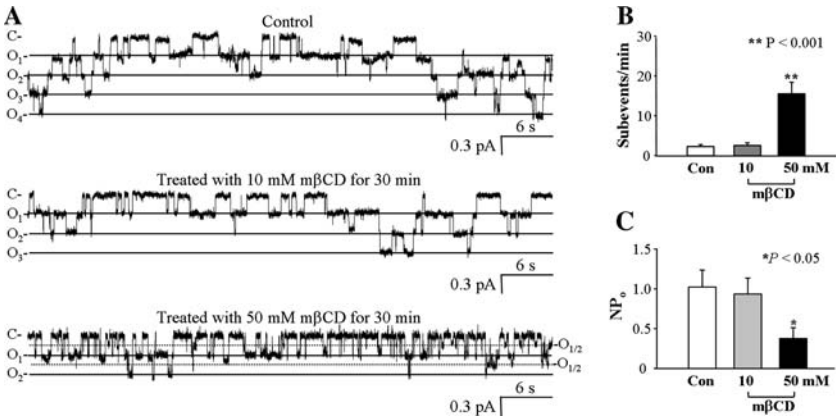

Fig. 4 A higher concentration of luminal mβCD alone increases ENaC activity and induces the appearance of subconductance. **a** Representative single-channel current from a control A6 cell (*upper trace*) and A6 cells pretreated with either 10 (*middle trace*) or 50 (*lower trace*) mM mβCD for 30 min. **b** Summary plots of ENaC subconductive openings (subevents) per minute in control cells (*white bar*) and cells pretreated with either 10 (*gray bar*) or 50 (*black bar*) mM mβCD for 30 min (2 ± 1 *vs.* 16 ± 3/min, $n = 6$, $P < 0.001$). **c** Summary plots of ENaC NP_o values in control cells (*white bar*) and cells pretreated with either 10 (*gray bar*) or 50 (*black bar*) mM mβCD for 30 min (1.02 ± 0.21 *vs.* 0.38 ± 0.14, $n = 8$, $P < 0.05$)

deposited on the surface of polyester membrane and diffused into the pores of the support material (lower right in Fig. 6).

Luminal Cholesterol at a Higher Concentration Mimics the Effect of 50 μg/ml Cholesterol Combined with Membrane Tension on ENaC Activity

If the tight packing of the outer leaflet of the apical membrane prevents cholesterol incorporation into the apical membrane, a high concentration of cholesterol alone may be able to produce the effect. To confirm what we found by using the back-filling approach, A6 cells were pretreated with cholesterol at two concentrations (50 and 200 μg/ml) for 30 min. Pretreatment with 200 μg/ml cholesterol, but not 50 μg/ml, increased the frequency of subconductive openings (subevents), as shown by a representative single-channel trace (Fig. 7a), from 2 ± 1/min (control, $n = 7$) to 31 ± 6/min (200 μg/ml cholesterol, $n = 7$, $P < 0.001$) (Fig. 7b) and increased ENaC NP_o from 0.58 ± 0.10 (control, $n = 7$) to 0.95 ± 0.12 (200 μg/ml cholesterol, $n = 7$, $P < 0.05$) (Fig. 7c). These data, together with the results from the back-filling approach, suggest that either membrane tension or a higher concentration is required for luminal cholesterol to increase both unitary and subconductive ENaC activity.

Discussion

Consistent with the results from short-circuit current experiments (West et al., 2005; Balut et al., 2006), our single-channel data demonstrate that luminal mβCD at 10 mM is unable to acutely affect ENaC activity at resting conditions. However, it is premature to conclude that ENaC is not regulated by apical cholesterol because apical or raft cholesterol is difficult to extracted by cyclodextrin and HDL (Remaley et al., 1998; Scheiffele et al., 1999). It is known that the cholesterol in the outer leaflet of the apical membrane is tightly packed with sphingolipids (Le et al., 1988; Remaley et al., 1998). Thus, maneuvers that could interfere with this tight packing should facilitate the extraction of apical cholesterol by mβCD. Since mechanical stretch affects not only lateral diffusivity (Svetina et al., 1998) but also the transbilayer migration of lipid molecules (Raphael & Waugh, 1996; Svetina et al., 1998), we hypothesized that mechanical stretch might permit luminal mβCD to regulate ENaC by facilitating its ability to extract apical cholesterol. This hypothesis is supported by the fact that mechanical perturbation facilitates scavenging of cellular cholesterol by HDL (Yancey et al., 1996). Our single-channel data have shown that when the cell membrane is under physical stretch, luminal mβCD strongly reduces ENaC activity.

The present study also shows that luminal cholesterol increases ENaC activity at both unitary and subconductive states. The latter effect appears to paradoxically mimic that of mβCD. However, this "paradoxical" effect nicely fits into an assumption that lipid rafts are required for ENaC to be fully open because atomic force microscopy studies show that lipid rafts can be deconstructed by both extraction and enrichment of cholesterol (Lawrence et al., 2003). Since the effect of cholesterol on ENaC conductance is dependent on mechanical stretch, just like that of mβCD, mechanical stretch may also be required for

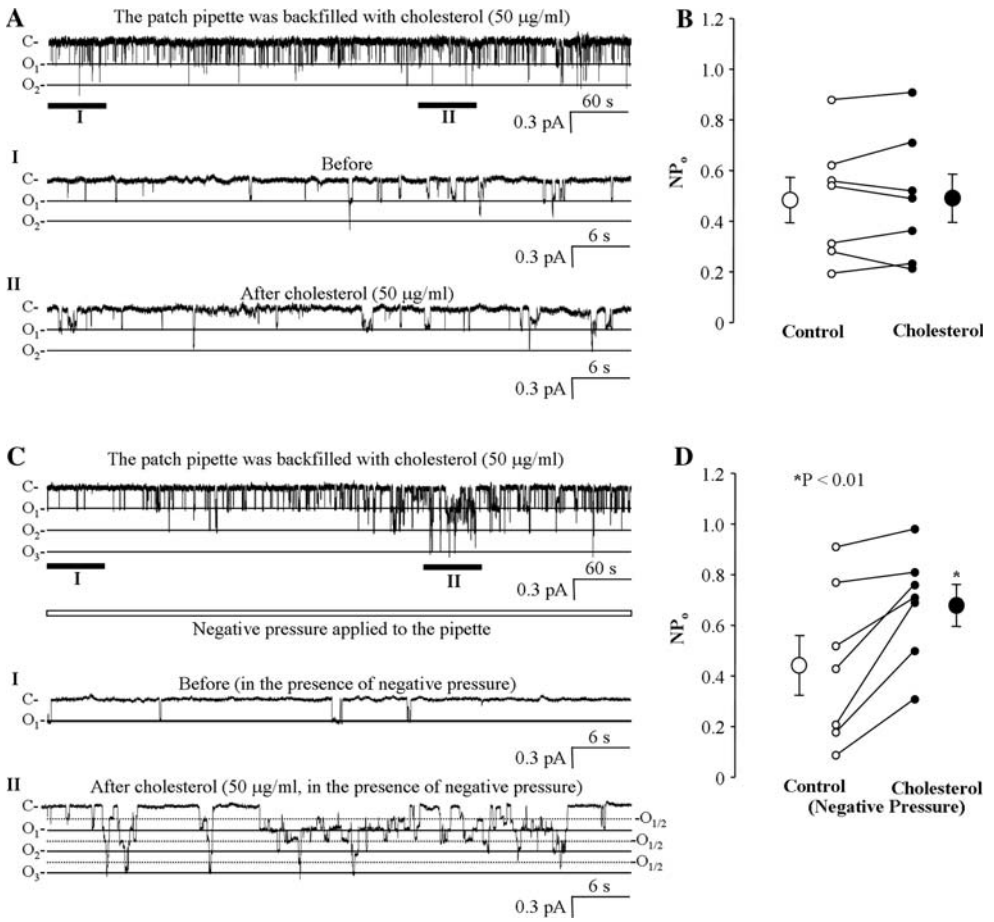

Fig. 5 Luminal cholesterol increases ENaC activity in the presence of negative pressure. **a** Representative single-channel current before and after application of cholesterol recorded from an A6 cell under control conditions. The patch pipette was back-filled with NaCl solution containing 50 μg/ml cholesterol. *I* shows a zoom-in period of control (before) ENaC activity, while *II* shows a zoom-in period of ENaC activity after cholesterol diffused down the pipette to the patch membrane. **b** Summary plots of NP_o values before (18–21 min after the patch pipette was back-filled with 50 μg/ml cholesterol) and after (26-30 min after filling) application of cholesterol under control conditions (0.48 ± 0.09 *vs.* 0.49 ± 0.10, $n = 7$, $P = 0.76$). **c** Representative single-channel current before and after cholesterol recorded from a cell-attached patch when constant negative pressure was applied to the pipette which was back-filled with NaCl solution containing 50 μg/ml cholesterol. **d** Summary plots of NP_o values before and after cholesterol in the presence of negative pressure (0.44 ± 0.12 *vs.* 0.68 ± 0.08, $n = 7$, $P < 0.01$). The same time frames as in **b** were used for calculating NP_o values

incorporation of cholesterol into the outer leaflet of the apical membrane to regulate ENaC. However, it remains to be investigated whether and why an intact lipid raft is important for ENaC to be fully open. Since the effects of mβCD and cholesterol on ENaC activity are opposite, it seems that, unlike its effect on ENaC conductance, the total amount of cholesterol in the apical membrane rather than lipid raft structure is positively correlated with ENaC activity. Since phosphatidylinositol 4,5-bisphosphate (PIP_2) also forms a cholesterol-dependent microdomain (Pike & Miller, 1998; Laux et al., 2000), one speculation is that enhanced cholesterol may further concentrate PIP_2 in a local area of the inner leaflet of the apical membrane where it stimulates ENaC (Ma, Saxena, & Warnock, 2002b; Yue et al., 2002; Tong & Stockand, 2005; Ma & Eaton, 2005).

Renal epithelial cells are always under membrane tension caused by either shear stress or the formation of fluid-filled cysts in polycystic kidney disease (Torres & Harris, 2006). HDL is found in the urine of patients with either glomerular diseases (Hotta et al., 2004) or nephrotic syndrome (Streather et al., 1993). Under these pathological conditions, cholesterol in the apical membrane might be extracted by HDL. Therefore, in these renal diseases, extraction of apical cholesterol by HDL may affect sodium absorption by reducing ENaC activity. In hypertensive patients with hypercholesterolemia, mechanical stretch from high blood pressure may facilitate cholesterol passage through the endothelial barrier to reach vascular smooth muscle cells. Since ENaC is also expressed in vascular smooth muscle cells and acts as a vascular mechanosensor for myogenic constriction (Benos, 2004; Drummond,

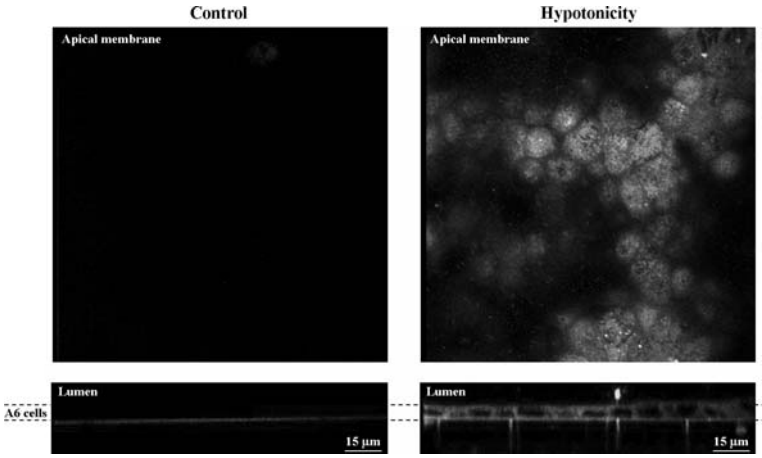

Fig. 6 Membrane tension is required for efficient uptake of luminal exogenous cholesterol by A6 cells. A6 cells cultured on polyester membrane attached to Transwell inserts were incubated either in NaCl solution (*Control, left*) or in hypotonic NaCl solution (*Hypotonicity*, *right*) for 7 min. NBD-cholesterol (50 μg/ml) was then added to the apical bath and the cells were incubated for 3 min more. *Square* and *rectangular images*, respectively, show confocal microscopy flat view of the apical membrane (*top*) and vertical sections (*bottom*) of A6 cell monolayer. The same gain for detecting fluorescence was used in each scan. The figure shows representative images from three consistent results

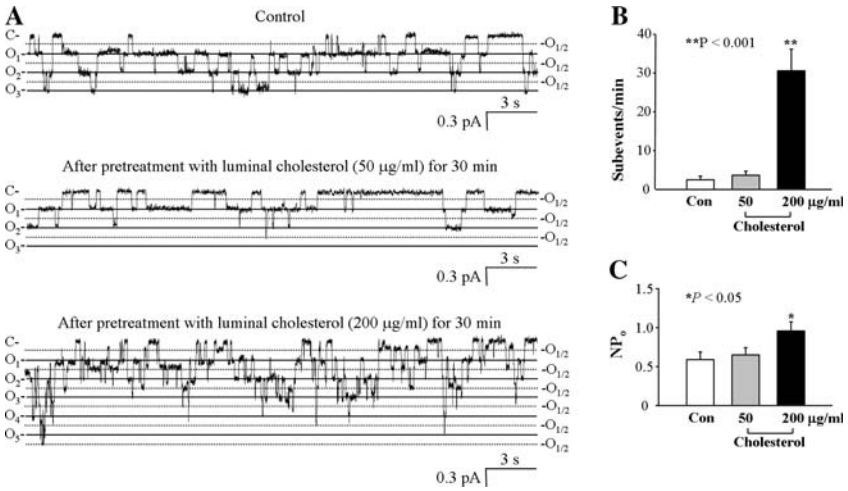

Fig. 7 A higher concentration of luminal cholesterol increases ENaC activity and induces the appearance of subconductance. **a** Representative single-channel current from a control A6 cell (*upper trace*) and A6 cells pretreated with either 50 (*middle trace*) or 200 (*lower trace*) μg/ml cholesterol for 30 min. **b** Summary plots of ENaC subconductive openings (subevents) per minute in control cells (*white bar*) and cells pretreated with either 50 (*gray bar*) or 200 (*black bar*) μg/ml cholesterol for 30 min (2 ± 1 *vs.* 31 ± 6/min, $n = 7$, $P < 0.001$). **c** Summary plots of ENaC NP_o values in control cells (*white bar*) and cells pretreated with either 50 (*gray bar*) or 200 (*black bar*) μg/ml cholesterol for 30 min (0.58 ± 0.10 *vs.* 0.95 ± 0.12, $n = 7$, $P < 0.05$)

Gebremedhin, & Harder, 2004; Jernigan & Drummond, 2005), the excessively absorbed cholesterol may serve as a complement of mechanical stretch to further myogenic constriction by stimulating ENaC. Studies related to these pure speculations may account for the clinical observations that cholesterol-lowering agents reduce blood pressure in hypertensive humans (Glorioso et al., 1999; Borghi et al., 2001, 2004).

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