Modulation of Basal and Peptide Hormone-Stimulated Na+ Transport by Membrane Cholesterol Content in the A6 Epithelial Cell Line

T. Aaron West and Bonnie L. Blazer-Yost

Biology Department, SL 358, Indiana University – Purdue University at Indianapolis

Abstract
These studies examined the effect of altering plasma membrane cholesterol on basal Na+ flux as well as on the natriferic responses to the peptide hormones, insulin and anti-diuretic hormone (ADH) in the A6 model renal cell line. Membrane cholesterol concentrations were depleted or enriched using methyl-β-cyclodextrin (MβCD) or a MβCD/cholesterol inclusion complex respectively. Effects of changes in the apical and basolateral plasma membranes were examined independently. Apical membrane cholesterol removal or supplementation had no effect on the basal Na+ transport rate. Short-term apical membrane cholesterol supplementation also had no effect on insulin-stimulated Na+ transport or on the initial phase of the ADH response. Interestingly, the additional apical membrane cholesterol had an inhibitory effect on the ADH response after 30 minutes. Apical membrane cholesterol depletion partially inhibited the responses to both insulin and ADH. Conversely, supplementation of basolateral cholesterol caused a significant increase in basal Na+ flux. Removal of cholesterol from the basolateral plasma membrane caused a decrease in basal Na+ flux with a time course analogous to channel turnover and completely inhibited peptide hormone responses. None of the changes in membrane cholesterol content decreased transcellular resistance. These results indicate an important role for membrane cholesterol content in the regulation of ENaC-mediated Na+ uptake.

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Introduction

Regulation of the epithelial Na+ channel (ENaC) is a major factor in the maintenance of salt and fluid homeostasis. ENaC is controlled by several hormonal systems in a tissue-specific manner. Interestingly, both steroid (aldosterone) and peptide (insulin and antidiuretic hormone, ADH) hormones appear to exert natriferic (salt retaining) effects via an increase in the number of channels in the apical membrane of polarized epithelial cells [1-6].

Bonnie L. Blazer-Yost, Ph.D.
Biology Department, SL 358
Indiana University – Purdue University at Indianapolis
723 West Michigan Street, Indianapolis, IN 46202 (USA)
Tel. +1 317-278-1145, Fax +1 317-274-2846, E-Mail bblazer@iupui.edu

In high resistance epithelial cells, binding of blood-borne peptide hormones to receptors on the basolateral membrane initiates complex intracellular signaling cascades that culminate with the insertion of ENaC into the apical membrane. Recent studies examining insulin-stimulated Na^+ transport have shown that an important facet of ENaC trafficking is the movement of the channel along the cytoplasmic leaflet of the basolateral membrane in response to diffusion of newly formed phosphoinositide lipid intermediates [5-6]. As these low abundance lipids of the inner leaflet diffuse from their point of synthesis in the basolateral membrane, cross the tight junctions and enter the apical membrane, the change in membrane environment appears to favor channel insertion. Concurrently, ENaC moves from sites adjacent to the lateral membrane and into the apical membrane [6]. The precise mechanisms governing these interactions are unknown but will likely involve additional membrane de-limited factors.

In addition to hormonal regulation, other factors appear to modulate channel function. Paracrine or autocrine effectors such as prostaglandins acutely regulate ENaC [7-8]. The concentration of the Na^+ ligand itself as well as extracellular Ca^{2+}, Mg^{2+} and osmolarity [9-12] also influence channel activity. Because of the documented importance of the ENaC in the maintenance of blood volume and, hence, blood pressure, it is likely that additional factors may also contribute to more subtle regulation of channel activity.

Recent epidemiologic studies have indicated a link between plasma cholesterol and hypertension. Cholesterol lowering agents, especially the statins, have been shown to lower blood pressure in hypertensive humans [13-15]. Cholesterol can partition into and out of the plasma membrane, particularly the basolateral membrane [16-18] and, therefore, it is likely that changes in plasma cholesterol will ultimately be associated with changes in the lipid composition of the membrane.

Because of the importance of membrane structure in the trafficking of ENaC, the current studies were designed to examine the effects of altering cholesterol on basal and peptide hormone-stimulated Na^+ transport in the A6 cell model of polarized epithelial cells. Interestingly, we demonstrate that the cholesterol content of the basolateral membrane may be a decisive factor in the level of Na^+ transport in these high resistance epithelial models of the principal cells of the distal tubule.

Materials and Methods

Cell Culture

The A6 model renal cell line derived from *Xenopus laevis* was used in these studies. The cells were fed three times a week with media containing; 7 parts Coon's F-12 medium, 3 parts Leibovitz's L-15 medium, 103 mM NaCl, 25 mM $NaHCO_3$, 25 U/ml penicillin, 25 µg/ml streptomycin, 1mM Glutamax (Invitrogen, Carsbad, CA), 10% newborn calf serum (ICN, Aurora, Ohio), pH 7.4. The cells were maintained in a humidified incubator at 27°C that was injected with 5% CO_2. Once cells reached confluence (~7 days), they were subcultured and seeded onto plastic flasks at one-tenth confluent density. For electrophysiology experiments, cells were seeded at one-third confluent density on nucleopore Transwell filters (4.7cm² Costar Transwells; Corning Costar, Cambridge, MA).

Electrophysiology

Functional ion transport of the A6 model renal cell line was measured as short-circuit current (SCC) [19]. Confluent cell monolayers (≥14 days after passage) seeded on nucleopore filters were removed from their Transwell chamber and inserted between the halves of a Ussing chamber. SCC measurements were taken from the Ussing chamber fitted with voltage and current electrodes located on both sides of the polarized epithelium. Cells were bathed in serum-free, non-supplemented medium which was circulated and oxygenated by means of a 5% CO_2-95% O_2 gas lift and water jacketed to maintain a constant temperature environment (27°C). The electrodes were connected to a current-voltage clamp apparatus for measurement of net ion transport under a zero voltage-clamp. Transepithelial resistance was measured every 200 secs by subjecting the monolayer to a 2 mV pulse. The resulting SCC deflection was used to calculate resistance by Ohms law. Only cell cultures exhibiting resistances greater than 1000 $\Omega \cdot cm^2$ were used. Once cells reached a stable baseline ion transport level, 0.5 mg/ml cholesterol or 10 mM methyl-β cyclodextrin (MβCD) were added to either the luminal or serosal side as indicated. After the 30 minute preincubation, hormones (100 mU/ml ADH or 30 nM insulin) were added to the serosal bathing media. To determine the amount of SCC due to Na^+ reabsorption, amiloride (10^{-5}M), an ENaC specific inhibitor, was added to the apical media 30 minutes after hormone stimulation.

Cholesterol Addition and Depletion

To add cholesterol to the plasma membrane of the A6 cell line, a MβCD/cholesterol inclusion complex was synthesized. Cholesterol (22 mg; Sigma Chemical Co, St. Louis, MO) was dissolved in 20 mls of ethanol and MβCD (478 mg) (Sigma) was added to the cholesterol solution and stirred for 20 minutes. The ethanol was evaporated in a vacuum oven at 45 - 50°C and 75 kPa for 1 hour until inclusion complex crystals formed. Serum-free media (2.2 mL) was added to the inclusion complex crystals and stirred until fully dissolved. Once the epithelia stabilized in the Ussing chamber, 1mL of the bathing media was removed from the cell bath and replaced with 1 ml of the inclusion complex containing serum-free media for a final concentration of 0.5mg/ml cholesterol. The data obtained using the inclusion complex

were compared with electrophysiological studies performed using a commercially prepared inclusion complex (Sigma #C4951, soluble cholesterol). Identical results were obtained using either complex (data not shown).

For cholesterol depletion from the membrane, a 100 mM MβCD solution was made in serum-free media. For electrophysiology experiments, 1 mL of the bathing serum-free media was replaced with 1mL of the MβCD solution for a final concentration of 10 mM.

Results

It is difficult to accurately predict the concentration of cholesterol present at the external face of the plasma membrane. The cholesterol concentration at the basolateral cell surface will be the result of complex interactions between the cholesterol carriers in the bloodstream as well as diffusion across the various layers of the vasculature. Therefore we have chosen to use a concentration of cholesterol (0.5 mg/ml) which is lower than the normal plasma levels.

Methyl-β-cyclodextrin (MβCD) is a reagent that can be used to effectively deplete or enrich cholesterol content of cellular membranes. MβCD is an efficient cholesterol acceptor which allows cholesterol molecules to desorb from the plasma membrane surface and to diffuse into the hydrophobic core of the cyclodextrin molecule [18, 20]. Conversely, when MβCD is used as a cholesterol carrier, the cholesterol will partition from a MβCD/cholesterol complex into the plasma membrane according to concentration gradients. The small size of the complex allows for an alignment adjacent to the cell membrane thus providing an environment for partitioning without transition into the aqueous phase [20].

Short-term (30 minute) incubation with a MβCD/cholesterol complex (soluble cholesterol) or MβCD alone was used to, respectively, enrich or deplete the plasma membrane cholesterol content of polarized A6 cell monolayers. Culturing the cells on permeable supports provides a fully polarized, high resistance phenotype and allows for the addition of reagents to a specific side of the monolayer. A6 cells exhibit a basal level of ion transport which is predominately due to reabsorptive Na$^+$ flux [21]. Addition of cholesterol to the apical face of the cells for 30 minutes was without effect on the basal Na$^+$ transport rate. A similar addition to the serosal face of the cells however, significantly enhanced the basal transport rate (Fig. 1).

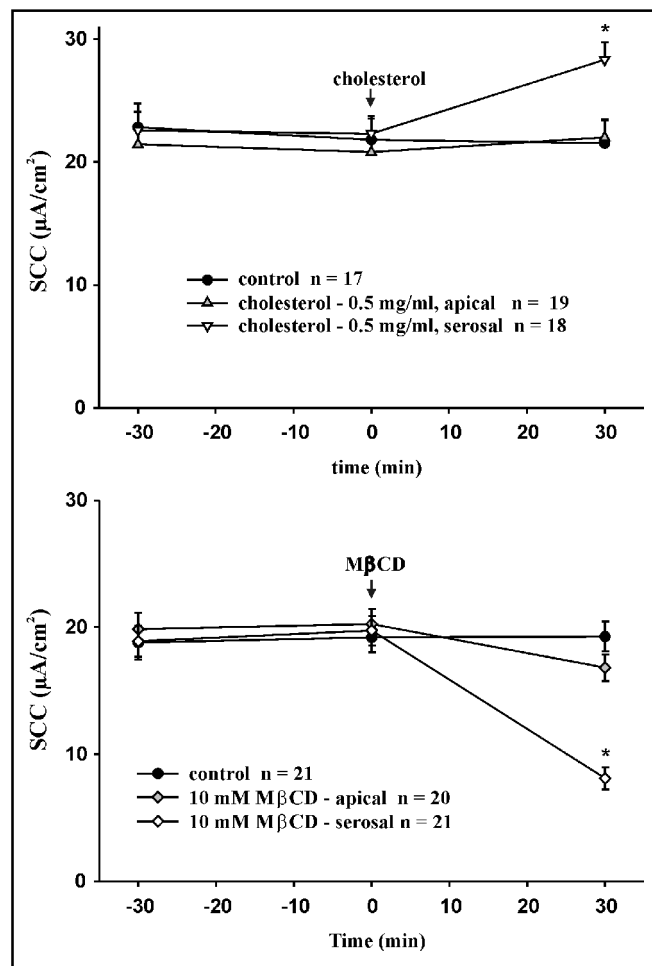Partitioning of cholesterol out of the apical cell membrane with MβCD had a very slight (non-significant)

Fig. 1. Ion transport effects of cholesterol enrichment or depletion from the apical and basolateral plasma membranes of A6 cells. A6 cells were grown to confluence on permeable filter supports. Net ion transport was monitored using the electrophysiological technique of short-circuit current (SCC). Cholesterol was added as a MβCD/cholesterol complex; cholesterol was depleted using MβCD alone. Symbols represent means ± SEM. Addition (top panel) or depletion (bottom panel) of cholesterol in the apical membrane had no significant effect on SCC while addition or depletion of cholesterol in the basolateral membrane significantly (P < 0.01) altered ion transport.

inhibitory effect on basal Na$^+$ flux. However, removing cholesterol from the basolateral membrane dramatically lowered the basal ion flux (Fig. 1).

To ascertain whether the changes in cholesterol content had effects on membrane integrity or tight junction permeability, the transcellular resistance were compared before and after the changes in plasma membrane cholesterol content. Figure 2 shows that addition or
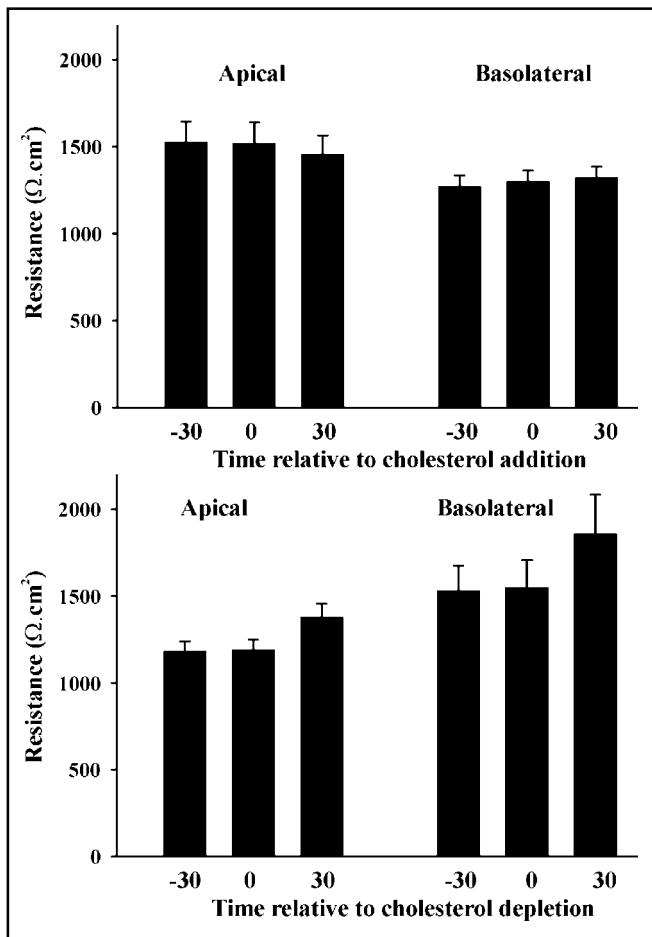
Fig. 2. Transepithelial resistance measurements in A6 cells after enrichment or depletion of cholesterol from the apical and basolateral plasma membranes. Time indicated in the figures is relative to the cholesterol addition (top) or depletion (bottom). Bars indicate means ± SEM of 13 experiments in the enrichment experiments and 12 experiments in the depletion experiments.

Fig. 3. Insulin-stimulated Na$^+$ transport in A6 cells after enrichment or depletion of cholesterol from the apical and basolateral plasma membranes. A6 cells were grown to confluence on permeable filter supports. Ion transport was monitored using the electrophysiological technique of short-circuit current (SCC). Cholesterol was added as a M?CD/cholesterol complex; cholesterol was depleted using MβCD alone. Cholesterol modifying agents were added at time t = -30 min. Insulin (30 nM) was added to all cultures at time zero. Amiloride (10^{-5}M) was added to all experimental conditions at time t = 30 min. Symbols represent means ± SEM.

depletion of cholesterol from either the apical or basolateral cell membranes did not change the transcellular resistance of the epithelial monolayer.

The A6 cells respond to various natriferic hormones including insulin and ADH. Addition of insulin to cells which had been supplemented with cholesterol on either the apical or basolateral face of the plasma membrane resulted in an increase in transcellular Na$^+$ flux, the magnitude of which was not different from the untreated cells. Since the insulin response was the same in samples where the cells had an already elevated level of basal transport due to the serosal addition of cholesterol, the
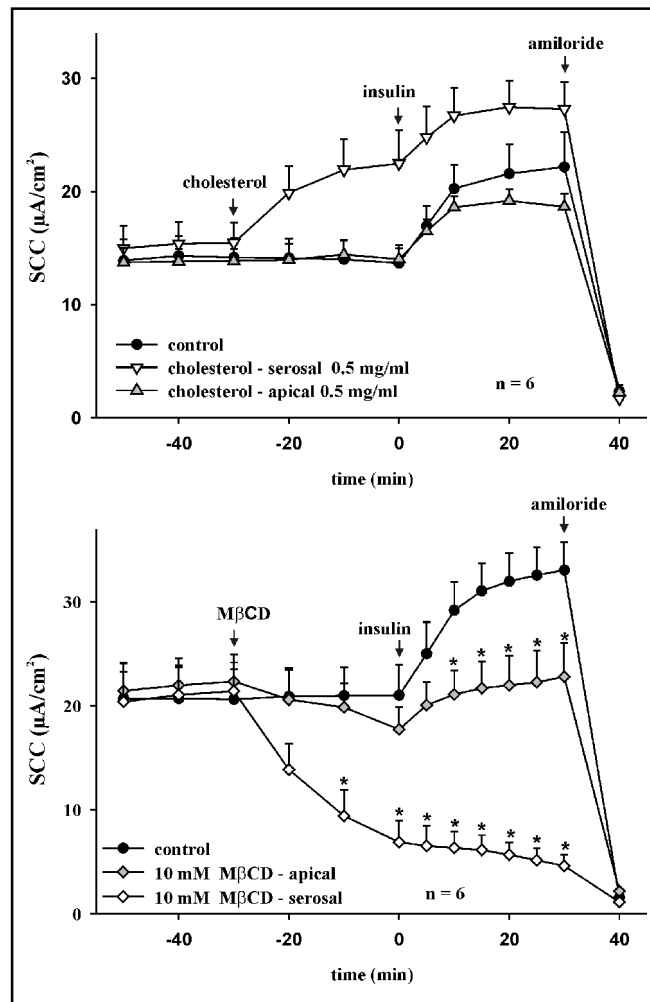
effect of cholesterol plus insulin resulted in an enhanced Na$^+$ transport response (Fig. 3, top panel)

Removal of cholesterol with MβCD had the opposite effect on the response to insulin. Depletion of cholesterol from the apical face of the cells diminished, but did not abolish, insulin-stimulated Na$^+$ transport. Depletion of cholesterol from the serosal plasma membrane completely

Fig. 4. ADH-stimulated Na⁺ transport in A6 ▷ cells after enrichment or depletion of cholesterol from the apical and basolateral plasma membranes. A6 cells were grown to confluence on permeable filter supports. Ion transport was monitored using the electrophysiological technique of short-circuit current (SCC). Cholesterol was added as a MβCD/cholesterol complex; cholesterol was depleted using MβCD alone. Cholesterol modifying agents were added at time t = -30 min. ADH (100 mU/ml) was added at time zero to all cultures. Amiloride (10^{-5}M) was added to all experimental conditions at time t = 30 min. Symbols represent means ± SEM.
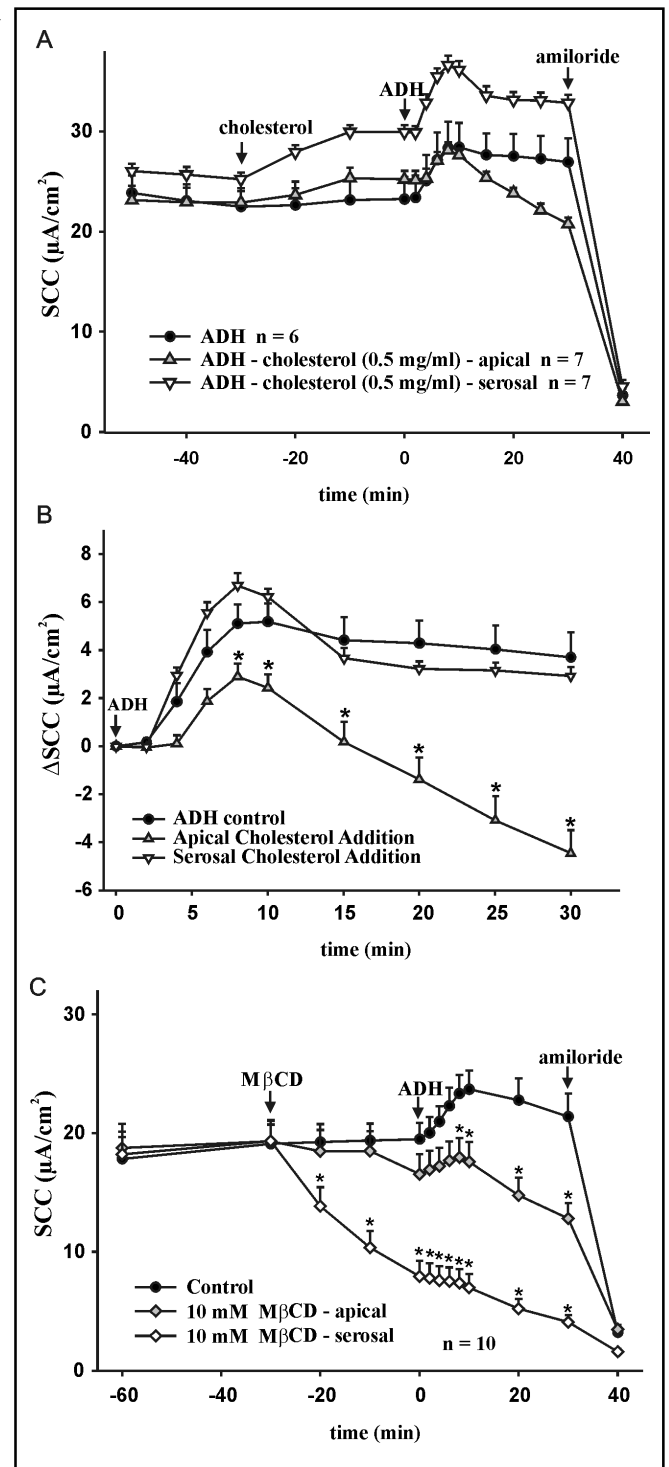

inhibited a subsequent response to insulin (Fig. 3, bottom panel).

Changes in plasma membrane cholesterol content also influenced the natriferic response to ADH (Figure 4). Cholesterol addition did not significantly alter the initial (first 10 minutes) magnitude of the response to ADH. As with the insulin response, the serosal addition of cholesterol followed by stimulation with ADH produced a Na⁺ transport response that reflected an additive response to the two factors. Interestingly, however, after the initial increase in transport, the cells which had additional cholesterol added to the apical membrane demonstrated a rapid decline in ADH-stimulated transport which resulted in a significantly lower level of amiloride-sensitive current within 30 minutes after ADH stimulation.

Removal of cholesterol with MβCD affected the ADH-stimulated ion transport response much like the insulin response. Cholesterol removal from the apical membrane partially inhibited a subsequent response to ADH while removal of cholesterol from the basolateral membrane completely abolished the ADH response (fig. 4).

Discussion

From a translational medicine standpoint, the most interesting observation drawn from the data presented here is that increased cholesterol on the basolateral side of the cell - the normal route for excess cholesterol to enter the cell membrane - leads to an increase in ENaC activity. This could be a contributing factor to the strong correlation between hypercholesterolemia and hypertension and may explain the decrease in blood

pressure that has been noted with statin use in hypertensive patients [13-15].

It is also important to note that the natriferic responses to peptide hormones are manifested in addition to the cholesterol-induced increase in Na^+ flux in a reabsorptive direction. Hormonal regulation via the factors in the renin-angiotensin-aldosterone axis such as aldosterone and ADH are modulated in response to blood pressure and, therefore, might exhibit compensatory changes. However, there are other factors which will adversely contribute to further increases in blood pressure. Metabolic Syndrome, affecting a growing number of people in Westernized society, is characterized by obesity, insulin resistance and compensatory hyperinsulinemia, dislipidemia and hypertension. The increased insulin together with an increased cholesterol undoubtedly contribute to the accompanying hypertension.

From a mechanistic viewpoint, the role of cholesterol in the regulation of ion transport is complex, particularly when considering the potential role for this lipid in the apical membrane, the site of active ENaC protein. Cholesterol is an integral component of "lipid rafts" or highly ordered lipid domains containing glycosphingolipids, sphingomyelin and cholesterol which preferentially attract particular types of proteins [22]. These rafts are usually envisioned as floating in the more abundant fluid phase lipid bilayer. Currently a rather popular view is that ion channels, particularly those in very low abundance, may be assembled with their regulatory components in lipid rafts. Functionally, rafts are often identified by their Triton X-100 insolubility or by "flotation assays" in which the low density rafts exhibit high buoyancy during density gradient centrifugation.

Hill et al.[23] used buoyant density measurements of endogenous ENaC in A6 cells to demonstrate that a fraction of the ENaC is present in the low density fraction (presumptively lipid rafts) and that a component of the low density fraction was located in the apical membrane. However, no functional measurements of Na^+ transport were performed in conjunction with these studies. Hanwell and colleagues [24] functionally expressed ENaC in another polarized epithelial cell line, the Madin-Darby canine kidney cells. In contrast to the previous results, these investigators found that both the intracellular and cell surface pools of ENaC were Triton X-100 soluble. Furthermore, buoyant density analysis indicated that these channels were not found in lipid rafts. Like Hill et al. [23], Shlyonsky and colleagues found ENaC isolated from A6 cells in both high and low density fractions with the active form of the channel found predominately in the low density fraction [25]. Despite this distribution, agents which remove cholesterol and sphingomyelin, presumably from the apical membrane, had no effect on channel activity.

When applied to polarized cells, the raft concept of small, defined areas of glycospingolipid-cholesterol-rich ordered domains may be an oversimplification. The lipid structure of the basolateral membrane is similar to that of unpolarized cells [16, 17, 26]. Furthermore, the lipids of the inner leaflet can diffuse across tight junctions [27, 28] and, by default, the cytoplasmic face of the apical membrane will have a lipid composition similar to that of the basolateral membrane. However, the external leaflet of the apical membrane is unique [16, 17, 26]. This leaflet is rich in glycosphingolipid and cholesterol and depleted in phospholipids and, in essence, could be considered as one large raft.

Our results indicate that cholesterol-dependent lipid rafts do not play a major role in the functional kinetic regulation of ENaC. Adding cholesterol to the apical membrane has no effect on basal or insulin-stimulated transport and, rather surprisingly, is inhibitory to a prolonged (>10 minute) ADH response. Apical removal of cholesterol has little effect on basal Na^+ transport but does appear to exert a partial inhibitory effect on insulin- and ADH-stimulated transport. Recent work from Balut and colleagues [29] has shown that removal of cholesterol from the apical membrane decreases to P_o of ENaC, a finding that is consistent with these studies. Since both hormones work via the insertion of new channels into the apical membrane [4-6] our results suggest that the cholesterol content of the membrane may be important for the insertional event and, subsequently, for regulation of the open probability ENaC in the apical membrane.

In the basolateral membrane, the cholesterol content is of prime importance for ENaC -mediated Na^+ transport. Removal of cholesterol from the basolateral membrane leads to a decline in basal transport and a complete inhibition of natriferic responses to either insulin or ADH. Several potential explanations could explain this finding. The most trivial is that the removal of cholesterol causes a loss of cell viability and/or integrity of the tight junctions. Both of these scenarios are detectable as a loss of transepithelial resistance. The transepithelial resistances of the epithelial monolayers were measured before and after cholesterol addition and depletion. Removing cholesterol from the apical or the basolateral membrane did not decrease the cellular resistance. Thus both cell

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viability and junctional integrity appear uncompromised by cholesterol depletion.

One possibility is suggested by the rate of decline in the basal transport rate as cholesterol is removed from the basolateral membrane. This decline in transport exhibits a time course very similar to that reported for the turnover of ENaC channels in the apical membrane of this cell type [30]. The effect on basal transport, together with the inhibition of insulin- and ADH-stimulated Na$^+$ transport would be expected if cholesterol were necessary for the trafficking and/or insertion of ENaC into the apical membrane.

We have shown that during a natriferic response to insulin, ENaC moves to the lateral membrane and appears to track along the inner surface of the membrane without entering the bilayer. In conjunction with an increase in phosphatidylinositol 3,4,5 trisphosphate diffusion from the lateral to the apical membrane, ENaC enters the apical membrane bilayer. It is possible that cholesterol plays an integral role in trafficking along the lateral membrane.

Recent studies have shown that cholesterol is part of several types of microdomains, only one of which displays the Triton X-100 insolubility which has become a hallmark of lipid rafts. Lang et al [31] have found that SNARE proteins such as syntaxins are clustered in a cholesterol dependent manner in PC12 neurosecretory cells. Despite the dependence on cholesterol, the syntaxin clusters are Triton X-100 soluble indicating that they are distinct from typical rafts. The clustering is necessary for exocytic fusion events and cholesterol depletion inhibits the rate of secretion. Inhibition of exocytosis by cholesterol depletion is consistent with our observations in the A6 cell line. PI3-kinase activity is necessary for hormone-stimulated Na$^+$ transport as well as for maintenance of basal transport [3, 32]. Recent data have suggested that in certain signaling pathways, cholesterol may be required for PI3-kinase activation [33].

In addition to potential effect on protein trafficking of transporters destined for the apical membrane, cholesterol may also exert direct effects on basolaterally located transporters, particularly the Na$^+$K$^+$ATPase [29]. The ATPase is not considered to be rate-limiting for Na$^+$ transport, particularly under un-stimulated conditions but inhibition of the important transporter would affect transcellular Na$^+$ transport. While the final details of the intracellular signaling pathways remain incompletely defined, our current results indicate that cholesterol is a necessary component of the basolateral membrane in A6 cells during hormone-stimulated Na$^+$ transport as well as for the maintenance of a basal transport rate.

Acknowledgements

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