The Epithelial Sodium Channel (ENaC) Traffics to Apical Membrane in Lipid Rafts in Mouse Cortical Collecting Duct Cells*

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Warren G. Hill1, Michael B. Butterworth1, Huamin Wang2, Robert S. Edinger3, Jonathan Lebowitz4, Kathryn W. Peters5, Raymond A. Frizzell1, and John P. Johnson6

From the 1Division of Matrix Biology, Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215 and the 2Department of Cell Biology and Physiology and the 3Laboratory of Epithelial Cell Biology, Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

We previously showed that ENaC is present in lipid rafts in A6 cells, a Xenopus kidney cell line. We now demonstrate that ENaC can be detected in lipid rafts in mouse cortical collecting duct (MPCcCD14) cells by detergent insolubility, buoyancy on density gradients using two distinct approaches, and colocalization with caveolin 1. Less than 30% of ENaC subunits were found in raft fractions. The channel subunits also colocalized on sucrose gradients with known vesicle targeting and fusion proteins syntaxin 1A, Vamp 2, and SNAP23. Hormonal stimulation of ENaC activity by either forskolin or aldosterone, short or long term, did not alter the lipid raft distribution of ENaC. Methyl-β-cyclodextrin added apically to MPCcCD14 cells resulted in a slow decline in amiloride-sensitive sodium transport with short circuit current reductions of 38.1 ± 9.6% after 60 min. The slow decline in ENaC activity in response to apical cyclodextrin was identical to the rate of decline seen when protein synthesis was inhibited by cycloheximide. Apical biotinylation of MPCcCD14 cells confirmed the loss of ENaC at the cell surface following cyclodextrin treatment. Acute stimulation of the recycling pool of ENaC was unaffected by apical cyclodextrin application. Expression of dominant negative caveolin isoforms (CAV1-eGFP and CAV3-DGV) which disrupt caveolae, reduced basal ENaC currents by 72.3 and 78.2%, respectively; but, as with cyclodextrin treatment, the acute response to forskolin was unaffected. We conclude that ENaC is present in and regulated by lipid rafts. The data are consistent with a model in which rafts mediate the constitutive apical delivery of ENaC.

Rafts are discrete transitory lipid domains that exist within the plane of cell membranes and that segregate lipids and proteins into regions that differ in their physical properties. They possess high concentrations of cholesterol and saturated sphingolipids in the outer leaflet of the bilayer, which contributes to a thicker, more closely packed liquid-ordered (lβ) phase thought to offer a thermodynamically favorable environment for certain membrane-associated and membrane-embedded proteins. In particular, GPI-anchored proteins and multiple acylated proteins such as the Src-like tyrosine kinases appear to have a high affinity for the more ordered lipid environment found in raft domains, principally as a function of their lipid modifications.

However, there is now a growing literature to show that integral membrane proteins such as transporters and ion channels can also be found in rafts. These include aquaporin water channels (1–3), CFTR (4), N-type Ca2+ channels (5), NHE3 (6), cardiac Na+ channels (7, 8), and a broad range of voltage-gated, inwardly rectified and Ca2+-activated potassium channels (reviewed in Ref. 9, 10).

Raft localization may have varying functional consequences: rafts are thought to operate as apical targeting platforms in the biosynthetic pathway, as organizers of receptor/signaling complexes on the cell surface or as sites of non-clathrin mediated endocytosis (11). In some cases rafts and/or cholesterol have been found to have functional effects on channel kinetics independent of trafficking, suggesting a role for protein-lipid interactions in modulating channel activities (12). We have previously shown that the epithelial Na+ channel (ENaC)3 is present in lipid rafts in A6 renal epithelial cells, which endogenously express this channel, and suggested that raft distribution was related to hormonal regulation (13). Subsequent studies in A6 cells have suggested that ENaC single channel kinetics are not directly affected by cholesterol depletion, although acute responses to hormonal stimuli are modestly reduced (14).

ENaC is expressed in epithelia which generate steep lumens to blood gradients and plays a critical role in regulating blood pressure through effects on sodium and fluid volume homeostasis. It is a heteromeric channel comprised of three homologous subunits (α, β, and γ) and is localized to the apical membrane of Na+ reabsorbing epithelial cells. Only a minority of ENaC subunits that are synthesized within a natively expressing cell appear to reach the apical membrane (15). The mechanism

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2 Both authors contributed equally to this study.

3 The abbreviations used are: ENaC, epithelial Na+ channel; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; m-CD, methyl-β-cyclodextrin.
of apical targeting is unknown. ENaC residence at the apical membrane is primarily regulated by ubiquitination, which is itself subject to hormonal regulation (16). The channel is internalized by clathrin-mediated endocytosis (17) and appears to be recycled at rates which are subject to hormonal regulation (18). ENaC activity is also regulated by proteolysis, some of which is related to post-translational processing within the protein secretory pathway and some of which appears to occur after apical membrane localization (18). The role of the lipid environment in processing, delivery, or regulation of channel function remains unclear.

To examine the role of lipid rafts in ENaC regulation we used a mammalian kidney cell line (mCCD cells, henceforth referred to as mCCD cells) and asked specifically whether endogenously expressed ENaC is present in lipid rafts and whether raft association is important for regulating its activity. We present data to show that ENaC is present in detergent-resistant and buoyant membranes in mCCD cells as well as in a membrane fraction prepared using techniques designed specifically to enrich for caveolin, a protein associated with lipid rafts. The role that raft localization may play in regulating ENaC expression is explored in the context of its colocalization with vesicular trafficking proteins. Our findings suggest that ENaC may utilize cholesterol-enriched membrane domains to traffic to the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—mCCD cells were allowed to polarize after subculturing onto permeable filter supports (0.4-μm pore size, 0.33 cm² surface area; Transwell, Corning Costar). Cells were grown on filters for at least 7 days in defined medium as described (18) prior to biochemical or electrophysiological analysis. Aldosterone was added at 1 μM for varying periods as described and in those experiments was grown in steroid-free media for 48 h prior to acute addition of aldosterone. Antibodies to α-, β-, and γ-ENaC subunits were rabbit polyclonal antibodies as previously described (18, 19). Additionally a γ-ENaC antibody purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) was employed which gave identical results. Caveolin monoclonal antibody was also from Santa Cruz. Syntaxin 1 mouse monoclonal antibody was obtained from Sigma and clathrin heavy chain antibody was the generous gift of Dr. Linton Traub and VAMP2 and SNAP 23 antibodies were rabbit polyclonals from Synaptic Systems, Gottingen, Germany. All other reagents were obtained from Sigma unless otherwise specified.

**Isolation of Lipid Rafts**—Triton X-100-soluble and -insoluble membranes were prepared as described (13). Rafts were also prepared in the absence of detergents on discontinuous sucrose gradients using the method of Song et al. (20) as described in detail (13). Briefly, cell lysates were scraped from filter supports into 2 ml of 500 mM sodium carbonate, pH 11, after washing three times with ice-cold PBS. Cells were lysed by passing through small bore needles and a postnuclear supernatant was isolated by low speed centrifugation. Samples were mixed with 90% sucrose in 25 mM MES, 150 mM NaCl, pH 6.5, and placed in a centrifuge tube. The sample was then overlaid with 4 ml of 35% and 4 ml of 5% sucrose before centrifugation at 190,000 × g for 18 h at 4 °C. Fractions (1 ml) were recovered, and the protein was extracted with chloroform/methanol (13). Total proteins from each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and probed with specific antibodies as described (13).

**Isolation of Caveolin-enriched Membranes**—Membranes were prepared according to the method of Smart et al. (21). In brief, a postnuclear supernatant (PNS) fraction was prepared from homogenized cells, loaded onto a 30% Percoll gradient (Amersham Biosciences, Piscataway, NJ), centrifuged at 84,000 × g for 35 min; crude plasma membranes recovered and sonicated; made to 23% (v/v) Optiprep (Accurate Chemical and Scientific Corp., Westbury, NY), overlaid with a linear 20–10% Optiprep gradient and subjected to centrifugation at 52,000 × g for 95 min. The top five fractions were recovered, made to ~20% Optiprep, overlaid with 5% Optiprep and centrifuged again at 52,000 × g for 95 min. Caveolin is enriched in fractions 2–3 of this final gradient.

**Na⁺ Current Measurements**—Equivalent short circuit currents (Isc) were measured before and after the addition of 10 μM amiloride using an electrode voltage ohmmeter (EVOM) from World Precision Instruments. Current was calculated using the potential difference across the filter divided by the resistance normalized to surface area to obtain current readings in μA/cm². The reported value is amiloride-sensitive current. Cells cultured on filter supports were mounted in modified Costar Ussing chambers, and the cultures were continuously short circuited with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance was measured by periodically applying a 2.5-mV bipolar pulse and calculated using Ohm’s law. The bathing Ringers solution was composed of 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂, and 10 mM glucose. Chambers were constantly gassed with a mixture of 95% O₂/5% CO₂ at 37 °C, which maintained the pH at 7.4. Amiloride (10 μM) was added to the apical cell surface at the end of each experiment.

**Biotinylation of Apical Membrane**—CCD cells were washed with ice-cold PBS plus Mg²⁺ and Ca²⁺ with agitation on ice for 3 × 5 min to remove media. The apical membrane was biotinylated using 0.5 mg/ml S-S-biotin (Pierce) in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na₂HPO₄, pH 9) for 20 min. The basolateral surface was incubated in medium containing FBS to prevent biotinylation. The biotinylation was quenched by adding FBS-containing medium on the apical surface. Monolayers were then washed three times with ice-cold PBS with agitation on ice, and the cells were harvested. Cells were lysed in cell lysis buffer (0.4% DCA, 1% Nonidet P-40, 50 mM EGTA, 10 mM Tris-Cl, pH 7.4) at room temperature for 10 min. Protein concentration of the post nuclear supernatant was determined, and 300 μg of protein was combined with streptavidin beads (Pierce) and incubated overnight at 4 °C. Samples from the streptavidin beads were collected in 2× sample buffer containing 10% β-mercaptoethanol and incubated for 20 min at room temperature. The protein was heated to 95 °C for 3 min and separated by SDS-PAGE and subjected to Western blot analysis with β-ENaC monoclonal antibody (Santa Cruz Biotechnologies) and visualized using chemiluminescence (PerkinElmer Life Sciences).
Transfection of Dominant Negative Caveolin Mutants into mCCD Cells—mCCD cells were seeded on 12-mm transwells (0.4-μm pore; Costar, Cambridge, MA) for 3–5 days prior to infection with recombinant adenovirus at a multiplicity of infection (m.o.i.) of 250 for Cav1-eGFP. Adenoviral vectors were prepared by Cre-lox recombination (22) in the University of Pittsburgh Viral Core under the direction of Dr. Paul Robbins. The cells were rinsed with calcium-free PBS containing 1 mM MgCl₂ (PBS-Mg²⁺). Subsequently, individual transwells were incubated for 1 h at 37 °C with 50–μl drops of PBS-Mg²⁺-containing virus, with an additional 150 μl of PBS-Mg²⁺/virus placed on the apical surface of the transwell. Samples were then returned to normal growth medium for 48 h before analysis. Cav3-DGV was subcloned into pcDNA3.1Hygro vector (Invitrogen) and transfected into mCCD cells using Lipofectamine 2000 (Invitrogen) as described (23).

RESULTS

To determine whether ENaC subunits were present in Triton-insoluble membranes in mammalian epithelial cells, polarized mCCD cells were scraped from filters, homogenized, and a PNS fraction obtained as described under “Experimental Procedures.” The PNS was made to 1% v/v Triton X-100 at 4 °C and incubated for 30 min before running on SDS-PAGE and immunoblotting with ENaC subunit-specific antibodies.

FIGURE 1. ENaC subunits are detergent-insoluble. Polarized mCCD cells were scraped from filters, homogenized, and a PNS fraction obtained as described under “Experimental Procedures.” The PNS was made to 1% v/v Triton X-100 at 4 °C and incubated for 30 min before running on SDS-PAGE and immunoblotting with ENaC subunit-specific antibodies.

We next examined the distribution of ENaC in membranes separated on density gradients to determine whether sodium channel subunits could be found in membranes of high buoyancy prepared without detergent. This had been shown in A6 cells where our results suggested that aldosterone might alter the distribution of ENaC subunits between buoyant and higher density membranes (13). Cells were either stimulated with aldosterone (1 μM for 18 h, indicated by +) or left untreated (−) and were then lysed and membranes separated by density on sucrose gradients. Fractions from the gradients were chloroform/methanol-precipitated and then immunoblotted with antibodies to ENaC subunits, clathrin heavy chain protein (a non-raft marker) and caveolin-1, a marker of lipid rafts (Fig. 2). Low-density membranes which correspond to lipid rafts are found near the top of the gradient in fractions 4, 5, and 6 while non-raft markers are found at higher sucrose densities in fractions 8–12. The majority of caveolin-1 is found in fraction 5 while clathrin is entirely excluded from lipid raft fractions. Each of the ENaC subunits were found in raft fractions demonstrating that a proportion of ENaC resides within membranes with the buoyancy characteristics of lipid rafts or caveolae. Immunoblots with γ-ENaC antibody demonstrated both full-length (FL) and processed or cleaved (CL) subunits similar to what we (17) and others (24, 25) have described. Full-length and cleaved γ-ENaC subunits are found in both raft and non-raft fractions and their relative distribution is similar, suggesting that cleaved or processed forms of ENaC are not enriched in rafts. Quantitation of the distribution for unstimulated cells is shown in Fig. 3A and reveals no difference between cleaved and uncleaved. The antibody employed to detect α-ENaC in these studies did not detect a cleaved or processed form of this subunit.

The percentage of α-, β-, and γ-ENaC found in raft fractions are quantitated in Fig. 3B. This analysis revealed that a minority of the total cellular amount of each subunit appears in raft frac-
tions, but there is some difference in the relative distribution. The quantitation demonstrates that 33.2% of cellular α-ENaC appears in raft fractions compared with 14.6% of β- and 17.5% of γ-ENaC, respectively. The difference between α- and β-ENaC distribution in raft fractions was statistically significant (p < 0.05, n = 4–7), while the difference between α- and γ-ENaC subunit distribution did not reach significance.

Aldosterone-treated cells had higher total levels of ENaC (Fig. 2), but in contrast to our findings in A6 cells (13), the distribution between membrane domains did not appear to be altered by hormone treatment. Quantitation by densitometry of the effect of chronic aldosterone stimulation (1 μM, 16 h) confirmed that hormone treatment did not appear to shift ENaC into a chemically different membrane domain and this is true for γ-ENaC subunits whether comparing the distribution of full-length, cleaved, or total γ subunits (Fig. 4). The error bars in Fig. 4 are sufficiently large however, that it is possible there were subtle hormone-mediated shifts in ENaC localization, which were not revealed by this analysis.

A close comparison of the data for β-ENaC in Figs. 3B and 4 suggests there might be a difference between the two experiments, however, analyzing these data sets by Student’s t test revealed no statistically significant difference. These results indicate that in CCD cells at least, hormonal exposure to aldosterone does not alter the relative distribution of ENaC subunits between raft and non-raft membranes.

To further substantiate the presence of ENaC in rafts we used a second membrane isolation protocol specifically designed to isolate membranes enriched in caveolae (21). This technique employs a combination of Percoll and Optiprep gradients to isolate a membrane fraction which is 9.5–1.8-fold enriched for caveolin-1 compared with total plasma membrane (26). Percoll gradients were used to prepare crude membranes from a postnuclear supernatant, then light membranes were isolated from the top of a 10–20% Optiprep gradient (first 5 of 12 fractions) and finally those membranes were concentrated on a second discontinuous Optiprep gradient. Membranes in fractions 1–6 from the top of the final gradient were concentrated by pelleting at 100,000 g for 30 min and then immunoblotted for ENaC and caveolin. Fig. 5 illustrates the presence of all three ENaC subunits in caveolin-1 positive fractions thereby confirming under different separation conditions that ENaC is present in membrane rafts.

We also used this preparation to further examine the question of whether aldosterone treatment at earlier time courses might alter distribution of ENaC subunits within membrane domains as our data in A6 cells had suggested (13). Treatment of mCCD cells with 1 μM aldosterone for 4 h results in stimulation of ENaC activity without the increase in cellular ENaC subunit abundance noted with longer term treatment (19). The
early aldosterone response is, at least partially, a trafficking phenomenon related to induction of SGK1 and the phosphorylation of Nedd 4–2 resulting in enhanced ENaC expression at the apical membrane prior to synthesis of new subunits (27). Samples were separated on sequential Optiprep gradients as described under “Experimental Procedures,” and the most buoyant fractions are located in the first five lanes of the final discontinuous gradient, which is enriched in caveolin. Fig. 6A shows the distribution of α-, β-, and γ-ENaC in fractions 6–10 of the first Optiprep gradient. These fractions represent the heavy or denser membranes which remain after lighter membranes are removed for concentration on a subsequent gradient. It can be seen that aldosterone treatment has no effect on ENaC in these membranes. Fractions 1–5 from these same gradients are run once more on a discontinuous Optiprep gradient to concentrate caveolin and it can be seen in Fig. 6B that there is little difference in the amount of each subunit localizing to the caveolin-enriched membranes (2 and 3). Quantitation by densitometry revealed no statistically significant differences in ENaC distribution (Student’s t test, n = 3). Therefore we observe no discernible alteration in the membrane domain distribution of ENaC with aldosterone either early or late in its course of action using two different gradient systems.

In addition to regulation of ENaC by aldosterone, we have shown that stimulation of mCCD cells with forskolin results in an acute up-regulation of apical membrane ENaC expression and activity through an exocytic mechanism (18). Because recent studies have suggested that cholesterol depletion with methyl-β-cyclodextrin (m-CD) may affect acute stimulation of ENaC in A6 cells (14, 28), we examined the effect of forskolin stimulation on the distribution of ENaC subunits in raft and non-raft membranes from mCCD cells. Cells grown on filter supports were exposed to 10 μM forskolin from the basolateral side for 30 min, resulting in an increase in amiloride-sensitive $I_{sc}$ of 100%. Cells were harvested, lysed, and membranes separated on sucrose gradients as described. As shown in Fig. 7, acute stimulation of ENaC activity by forskolin had no effect on the distribution of α-ENaC subunits between raft and non-raft fractions of the gradient. These results suggest that up-regulation of ENaC resulting from enhanced expression and traffick-
ing of subunits to the apical membrane from intracellular compartments is not associated with major alterations in the membrane domains occupied by ENaC.

A number of transporters appear to use lipid rafts and lipid raft localized t-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and v-SNARE proteins as a means to direct their trafficking to the correct cellular compartment (29, 30). In a study of GLUT4 trafficking in 3T3-L1 adipocytes, SNAP23 and syntaxin 4 were associated with plasma membrane rafts while VAMP2 was primarily in intracellular lipid raft membranes. Upon insulin stimulation, VAMP2 redistributed to rafts within the plasma membrane and the membrane distribution of syntaxin and SNAP23 was unchanged (30). We therefore looked at whether SNARE complex proteins would colocalize to the same membrane fractions as ENaC. As seen in Fig. 8, syntaxin 1A, Vamp2, and SNAP23 primarily localized to lipid raft fractions (4–6) with the majority of SNAP23 and VAMP2 being raft localized. Syntaxin 1A exhibited significant partitioning to raft fractions as well as to heavier membrane fractions.

We employed two distinct approaches to investigate whether lipid raft localization was important for ENaC activity. To focus directly on raft-mediated phenomena at the cell surface we employed the cholesterol-sequestering drug m-CD. This reagent has been shown to disrupt rafts and to cause a dispersion of raft-localized proteins and lipids into the remaining (non-raft) plasma membrane (31). We applied m-CD to the apical and basolateral sides of polarized mCCD cells in a modified Ussing chamber and measured Na\(^+\) transport. Cells exposed to vehicle alone on the apical side (Fig. 9C) showed no change in amiloride sensitive Na\(^+\) transport over the duration of the experiment (black bars indicate addition of 10 \(\mu\)M amiloride). When 10 \(\mu\)M m-CD was added to the basolateral side (gray bar, Fig. 9A) there was a profound reduction in Na\(^+\) transport (79 ± 6\% after 60 min). This is almost certainly due to inhibitory effects on the activity of the basolateral Na-K-ATPase, which has been shown to be exquisitely sensitive to membrane composition and in particular to the level of membrane cholesterol (32, 33). Balut et al. (14) demonstrated dramatic reductions in Na\(^+\) transport in ENaC-expressing A6 cells upon basolateral treatment with m-CD. Prior treatment of the apical membrane with nystatin removed any rate limitation associated with Na\(^+\) entry into the cell, thus demonstrating the inhibition was most likely due to cholesterol-mediated effects on the Na\(^+\) pump. Cholesterol has also been shown to be critical for Na-K-ATPase activity in other cell types including renal cells (34). To verify that the effect was indeed localized to the ATPase on the basal membrane we performed a similar experiment in which we pretreated cells apically or basally with m-CD then permeabilized the apical membrane with nystatin and subsequently quantitated the amount of ouabain inhibitable current remaining. Fig. 10A shows a representative tracing of cells pretreated apically with m-CD while Fig. 10B summarizes the change in Isc from plateau to ouabain-inhibited \((n = 6\) and 4 for controls and m-CD-treated, respectively).

Apical application of 10 mM m-CD (gray bar, Fig. 9B) caused a slow progressive inhibition of Na\(^+\) transport and \(I_{sc}\) was 23.5 ± 5.6\% lower than control cells after 30 min and 38.1 ± 9.6\% lower than controls after 60 min. Although inhibition was more subtle than noted with basolateral addition, the effect was very reproducible. Given the slow kinetics of this effect it was tempting to speculate that cholesterol depletion and raft disruption were not having a direct effect on channel activity but may have altered delivery or retrieval of ENaC from the cell surface.

We have established a model of ENaC trafficking in mCCD cells (18) which involves a slow, constitutive turnover of newly synthesized channels which is sensitive to the protein synthesis inhibitor cycloheximide, and a more rapid recycling compartment which is largely insensitive to cycloheximide being largely composed of previously synthesized channel components. To determine which, if either, of these compartments might be associated with lipid rafts, we examined the effect of m-CD on both constitutive delivery and recycling. We first compared the effects of apical 10 mM m-CD to that of cycloheximide. As shown in Fig. 11, the effects of apical m-CD and cycloheximide on amiloride-sensitive \(I_{sc}\) were virtually identical, suggesting that disruption of lipid rafts by this agent has impaired delivery of channels to the apical membrane, reducing ENaC activity to
a similar extent as seen by blocking synthesis and delivery of new channels with cycloheximide. To confirm that the decline in ENaC activity was related to decreased surface expression, we measured apical membrane ENaC following 1 h exposure to apical m-CD using domain-specific biotinylation. As shown in Fig. 11, inset, ENaC expression at the apical membrane declined significantly (p < 0.001 by densitometry, n = 3). It should be noted that these data do not formally rule out the possibility of m-CD effects on endocytosis rather than exocytosis, i.e., that reduced Na transport may be due to enhanced internalization of ENaC. However several lines of evidence would seem to make this far less likely. It is known for instance that ENaC is endocytosed through clathrin-coated pits (17, 35) and that this process does not involve lipid rafts (36). Further, Luetal. (37) have confirmed ENaC endocytosis through clathrin-dependent route and specifically found that m-CD treatment did not affect ENaC internalization. These observations combined with the data shown in Fig. 11 strongly suggest that delivery of ENaC, not retrieval, is raft-dependent.

To determine whether disrupting rafts with m-CD alters the kinetics of the recycling pathway we repeated stimulation of the cells with forskolin under control conditions or following apical exposure to 10 mM m-CD. As shown in Fig. 12, forskolin stimulation results in increased Na transport (gray bars), which
then diminishes upon washout. It can be seen that repetitive stimulation of Na\(^+\) transport in response to forskolin remains intact under these conditions demonstrating that regulation of, and the kinetics of delivery and retrieval from the recycling pool, are not affected by raft disruption. These results suggest that lipid rafts may be required for trafficking of ENaC through the biosynthetic pathway but are less likely to be involved in recycling.

Given the possibility of nonspecific effects of m-CD, we chose to use a second completely different approach to disrupting rafts. We expressed dominant negative caveolin constructs and examined their effect on ENaC activity in mCCD cells. N-terminal modifications of caveolin appear to disrupt lipid rafts. Caveolin 1, N-terminally tagged with enhanced GFP, completely abrogates the endocytic function of caveoleae (38). Cav3DGV is an N-terminal deletion mutant lacking the first 53 amino acids of caveolin 3. This mutant disrupts caveolae and cholesterol-rich plasma membrane domains in a range of cell types including NIH3T3 cells (39), baby hamster kidney cells (39) and adipocytes (40) and in doing so reduces the number of morphologically distinct caveolae. Expression of both Cav1-eGFP and Cav3DGV resulted in significant inhibition of Na\(^+\) currents under both basal and forskolin-stimulated conditions. Data for Cav3DGV-expressing cells is shown in Fig. 13A. Basal amiloride-sensitive Na\(^+\) current was reduced by 78.2% in Cav3DGV-expressing cells. Total forskolin-stimulated current was reduced by 38.2% (Fig. 13A), but the degree of stimulation of control cells was 3.2-fold while in Cav3DGV cells forskolin evoked a 9.2-fold increase. The absolute increase in current was similar in both cases at 4.3 and 3.4 \(\mu\)A/cm\(^2\) for control and Cav3DGV cells implying that a comparable number of channels had been mobilized and inserted into the apical membrane (18, 41). Expression of Cav1-eGFP also resulted in a dramatic reduction in Na\(^+\) transport (Fig. 13B). In these experiments ENaC activity was reduced by 72.3%. These data indicate that perturbations to the cellular distribution of cholesterol and consequently to microdomains at the cell surface have inhibitory effects on whole cell Na\(^+\) currents but do not affect the acute regulation of ENaC trafficking. This is consistent with the m-CD data shown in Fig. 12.

**DISCUSSION**

Lipid rafts are thought to be important for trafficking in polarized epithelia and for signal transduction at the plasma membrane. Rafts form in the Golgi apparatus due to processes which segregate and enrich particular lipids into vesicular transport intermediates and are one element of many involved in the apical targeting machinery. The mechanisms which underlie the sorting of lipids and proteins in the biosynthetic pathway are not well understood, but proteins in rafts acquire detergent-insolubility (one of the biochemical hallmarks for raft localization) during their transit through the Golgi apparatus (42, 43). Depletion of cholesterol or disruption of sphingolipid synthesis has been shown to result in mislocalization of apical proteins to the basolateral domain (44, 45) thus underscoring the importance of the lipid component in vesicular traffic. Rafts also appear to preferentially segregate proteins into receptor/signaling modules at the plasma membrane. Rafts and non-raft membrane domains can spatially separate or conversely, physically colocalize proteins for functional interactions.

We and others (13, 46) previously demonstrated the presence of both total and cell surface ENaC in lipid rafts in endogenously expressing A6 cells. With the development of the lipid raft hypothesis and the concept of protein and lipid compartmentalization into discrete domains within the plane of the bilayer, there exists added potential for regulation of channel activity. Experiments in heterologous expression systems however, have produced conflicting results. Prince and Welsh (47) demonstrated detergent insolubility of ENaC in COS-7 and HEK293 cells, however Hanwell et al. (48) reported that ENaC was completely Triton X-100 soluble and was only found in membranes of high density when expressed in MDCK cells. The discrepancy between their study and ours in this respect is puzzling. Hanwell examined ENaC overexpressed in a cell line, which did not normally express it. It is possible that the relatively small fraction of subunits in rafts were not seen under these conditions, and it seems less likely to us that there would be a fundamental difference in apical trafficking of subunits between overexpression systems and endogenously expressing cells. We and others (46) have both described ENaC as being present in rafts in the only other endogenously expressing system examined (A6 cells). Secondly, they used a detergent-based method, and we used a specifically non-detergent based method for our gradient separations, and this may also have resulted in not recognizing a small fraction of raft-based ENaC. We have now extended our original observations by examining the membrane domain localization of ENaC in an endogenously expressing mammalian cell line. A fraction of ENaC is clearly present in lipid rafts in mCCD cells as shown by detergent insolubility, flotation analysis on sucrose and optiprep gradients and co-localization with caveolin-1. There are several possible reasons for the presence of ENaC in lipid rafts: rafts might represent a mechanism for delivery of the channel to the apical membrane; rafts could act as platforms for sequestering important regulatory molecules in close proximity to ENaC;
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rafts might serve as platforms for caveolar-mediated internalization and endocytosis, or the $I_n$, membrane environment of rafts might be important in regulating channel activity directly. The evidence presented here argues in favor of the first explanation and is consistent with one of the main tenets of the raft hypothesis.

SNARE proteins are universally conserved membrane fusion proteins whose role is to facilitate the correct targeting docking and fusion between membrane compartments. They fall into two classes (vesicle- or v-SNARES and target- or t-SNARES) and play a critical role in imposing specificity on membrane fusion events. We demonstrate here that the SNARE complex proteins, syntaxin 1A (plasma membrane), soluble NSF attachment protein 23 (SNAP23), and vesicle-associated membrane protein 2 (VAMP2) are found in lipid rafts in mCCD cells (Fig. 8). All three have previously been shown to be raft localized in MDCK cells and addition of anti-SNARE antibodies to permeabilized cells results in impaired apical trafficking of a reporter protein (49). In Xenopus oocytes, syntaxin 1A co-expression inhibited ENaC currents through a mechanism resulting primarily in reduced numbers of channels at the cell surface (50–52) but also through effects on $P_o$. Condliffe et al. (51) demonstrated direct protein-protein interactions between syntaxin and ENaC. Recently it was shown that SNAP23 also has an inhibitory effect on ENaC in HT29 colonic epithelial cells. SNAP23 interacts directly with ENaC and that interaction was augmented by syntaxin 1A in an oocyte expression system (53).

Thus there appears to be a complex interplay between these targeting proteins, resulting in fine tuning of $Na^+$ transport. In the light of these results and our own, we speculate that lipid raft compartmentalization of ENaC and SNARE complex proteins may provide a site for regulation of channel insertion into the plasma membrane. SNAP/SNARE proteins have been found to localize to lipid rafts in other cell types e.g. MDCK, PC12, 3T3-L1 adipocytes, RBL mast cells, and HeLa (reviewed in Ref. 54) whereas SNARE regulators like $\alpha$-SNAP, NSF, munc18, and complexin appear to exist in non-raft domains. Interestingly, SNARE regulators when complexed with SNARES are most often found in non-raft domains. This suggests that spatial separation of SNARES and their regulators facilitates exocytosis/membrane fusion and conversely, that regulated inhibition results in a shift in the SNARE complex out of rafts (54).

We have generated a model for ENaC trafficking in CCD cells based on measurement of channel activity over time following inhibition of protein synthesis, which suggests slow constitutive turnover of the channel at the apical membrane. In addition, the experiments demonstrate acute exocytic insertion of channels from a recycling pool following stimulation by forskolin. It seems reasonable to interpret the results of our experiments aimed at disrupting lipid rafts in light of this model. Application of apical membrane m-CD, which should result in cholesterol sequestration initially from apical membranes, results in only a slow and modest decline in ENaC current over a 1-h period exactly mirroring the time course of current decline seen with inhibition of new channel biosynthesis by cycloheximide, and this treatment is associated with a decreased expression of $\beta$-ENaC at the apical membrane. It is interesting that the acute response to forskolin stimulation, mediated by exocytic insertion from the recycling pool, was not affected by m-CD. We cannot rule out the possibility that there is a direct effect of m-CD on ENaC gating characteristics, but note that studies in A6 cells failed to show any direct effect of this agent on ENaC function, including gating characteristics measured by noise analysis or patch clamp (14). Disruption of lipid rafts by either constitutive expression of a dominant negative caveolin (Cav3DVG), or following 24-h expression of a dominant negative caveolin expressed via an adenoviral vector (Cav1-eGFP) had dramatic effects on ENaC function in CCD cells. Cav1-eGFP and Cav3DGV are N-terminal truncation mutants of caveolins which have been shown to reduce SV40 infectivity and the number of caveolae (38, 55). They also cause the intracellular accumulation of free cholesterol in late endosomes, a decrease in surface cholesterol and a decrease in cholesterol efflux and synthesis (56). Cav3DGV plays a role in disrupting overall cellular cholesterol homeostasis because cholesterol repletion restored all impaired cellular functions. Both dominant negative caveolin constructs resulted in substantial reductions in $Na^+$ currents, but, like m-CD, did not interfere with forskolin-stimulated increases in ENaC activity (Figs. 12 and 13). This implies that it is constitutive expression and turnover of ENaC that is impaired, while regulated recycling and acute exocytic responses are independent of raft organization.

The recent findings of Pelkmans and Zerial (57) add significantly to our understanding of caveolar trafficking dynamics. They show that caveolae exist at the plasma membrane as static multivesicular aggregates ("grape clusters") or as individual vesicles which undergo multiple kiss and run cycles from a small volume immediately adjacent to the cell surface. Under certain stimulatory conditions, caveolae escape this repetitive short range motion and can undertake long range intracellular transport to organelles now referred to as caveosomes (38, 57). Caveolin-1 may actually function as a negative regulator of lipid raft endocytosis, since reductions in caveolin-1 levels has been shown to have no effect or to accelerate internalization of other markers of caveolae (55). Therefore caveolae are unlikely to be active portals for ENaC endocytosis, which we have described as being primarily via a clathrin-dependent mechanism (17), and this suggests that the effect of dominant negative caveolins on ENaC activity is due to reduced channel numbers mediated through effects on constitutive delivery of channels to the apical membrane. This conclusion is supported by the m-CD experiment showing a slow loss of basal amiloride-sensitive sodium transport when cholesterol is removed from the apical membrane. If ENaC activity was being directly regulated by the physical properties of the bilayer, this intervention should have had both a faster and a more dramatic effect as was seen when m-CD was added basolaterally. The data from these experiments are best explained by a reduction in the constitutive delivery of ENaC to the plasma membrane with no effect on its acutely regulated insertion and retrieval.

In summary, a fraction of ENaC is present in and regulated by lipid rafts and the likely mechanism is through apically directed transport from the trans-Golgi network as a component of rafts. All of the necessary fusion and targeting machinery is
found in rafts and disruptions to cellular cholesterol metabolism have substantial effects on ENaC activity. Taken together, our findings strongly suggest that lipid rafts play a role in mediating constitutive exocytic trafficking of ENaC to the cell surface. We conclude that there is an intimate and heretofore unexpected link between cholesterol concentration, membrane domains and ENaC regulation.

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