The epithelial sodium channel (ENaC) present in the kidney collecting duct, distal colon, and the lung is responsible for salt reabsorption and whole body volume regulation. It is composed of three homologous subunits, α, β, and γ, which form a heteromultimeric protein likely to be comprised of two α subunits and one each of β and γ. These subunits are characterized by two membrane-spanning domains, short intracellular N and C termini, and a large ectodomain. In humans, mutations to this channel are known to be responsible for pseudohypoaldosteronism type I, a salt-wasting disease of infants, and Liddle’s syndrome, which results in severe hypertension. In the former, mutations to α, β, and γ result in loss-of-function due to lower channel density at the plasma membrane (2). Conversely, in the latter, deletions or mutations to PY motifs in the C termini of β or γ result in a gain-of-function which appears to result from increased residency time at the plasma membrane (3). Since both diseases result from altered trafficking and/or turnover of the channel it is of vital importance to understand the regulation of these processes. Recent studies from our laboratory have suggested that the trafficking and turnover of ENaC subunits may be regulated independently (4). Chronic aldosterone treatment of A6 cells, for example, dramatically increased levels of β at the cell surface concomitant with increases in Na⁺ conductance, while levels of α and γ remained unchanged. If channel subunits have distinctly different half-lives at the surface and if selective insertion and retrieval of channel subunits can regulate Na⁺ reabsorption, then biosynthesis and subunit trafficking are critically important to this process.

Membrane microdomains known as lipid rafts are enriched in cholesterol and sphingolipids and have been shown to exist as dynamic platforms important for the delivery of proteins to the apical membrane as well as for sequestering proteins in close physical proximity for functional interactions (5, 6). These structures are characterized by their detergent insolubility and high buoyant density. Since mature ENaC has been described as being detergent-insoluble when expressed in COS7 and HEK293 cells (7), we hypothesized that raft localization might represent a cellular mechanism for controlling ENaC subunit density at the plasma membrane and/or ENaC subunit interactions. In addition, a number of proteins known to interact with ENaC have been localized to lipid rafts, most notably the ubiquitin ligase NEDD4 (8), which plays a role in ENaC turnover at the plasma membrane (3). Therefore we have examined whether ENaC may be present in lipid rafts in the endogenously expressing A6 cell line. Our results indicate that all three subunits are present in lipid rafts and that the membrane microenvironment that ENaC exists within is altered by exposure to aldosterone.

EXPERIMENTAL PROCEDURES

Cell Culture—A6 cells were seeded at high density and grown on 75-mm permeable supports (0.4 µm pore, Costar, Cambridge, MA) or Millipore, Bedford, MA) in amphibian medium (BioWhittaker, Walkersville, MD) containing 10% fetal bovine serum. Cells were maintained at 28 °C in 5% CO₂ and were not used for experiments for at least 8 days. Current and resistance measurements across confluent monolayers were performed using an EVOM “chopstick” device (World Precision Instruments, Stevenage, UK). Cells were used for experiments if resistances were at least 800 Ω and currents of at least 1.5 µA/cm² were recorded.

Preparation of Triton X-100-soluble and -insoluble Membranes—Confluent cells with high resistances and Isc readings were scraped from filters into TNE buffer (in mM: Tris·HCl, 25; NaCl, 150; EDTA, 5; pH 7.4) containing protease inhibitors (PIs; Complete Mini, Roche

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lecular Biochemicals), disrupted by suction through a 22-gauge syringe and a post-nuclear supernatant (PNS) recovered after 1500 × g centrifugation for 5 min. Ice-cold Triton X-100 (final 1%) was added to the PNS for a 30 min. incubation on ice. Insoluble and soluble membranes were recovered following centrifugation (100,000 × g, 60 min), and insoluble membranes in the pellet were then dissolved in Triton X-100 and incubated at 28 °C for 30 min. Aliquots of soluble and insoluble membranes were added to sample buffer and boiled for 2 min prior to SDS-PAGE and Western blotting. Western blots were probed with ENaC subunit-specific antibodies as described in Refs. 9 and 10.

**Isolation of Membranes by Sucrose Gradient Centrifugation**—Membranes and different buoyant density were prepared essentially as described in Ref. 11. Briefly, cells were scraped into 2 ml of 500 mM sodium ENaC subunit-specific antibodies as described in Refs. 9 and 10. Insoluble membranes were added to sample buffer and boiled for 2 min before centrifugation at 190,000 × g for 18 h at 4 °C. Fractions (1 ml) were recovered, and protein was extracted with chloroform/methanol according to Ref. 12. Total proteins from each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and probed with ENaC subunit-specific antibodies as described (4). Xenopus caveolin was detected using an N-terminal specific antibody, which was raised against human caveolin-1 and cross-reacts with mouse and rat caveolin-1 (Santa Cruz Biotechnology, Santa Cruz, CA). To confirm that the band being detected was indeed caveolin, we immunoblotted A6 cell membranes that had been separated on sucrose density gradients with another commercially available antibody with reactivity to caveolin isoforms 1, 2, and 3 (BD PharMingen, Lexington, KY). This antibody recognized a band at 22 kDa, which floated in fractions 4 and 5 of the gradient and which ran with the same relative mobility as a positive caveolin control provided by the company (data not shown), thus confirming the specificity of the Santa Cruz antibody. Flotillin 2 antibody was obtained from Santa Cruz Biotechnology.

**Aldosterone Treatment**—Confluent transporting monolayers of A6 cells were treated with 1 μM aldosterone for 18 h at 28 °C.Sucrose gradient centrifugation was then performed as described above.

**Surface Biotinylation**—Cells were washed three times with cold PBS and a total of 2.5 mg sulfo-NHS-SS-biotin (Pierce) in a volume of 5 ml of ice-cold borate buffer (in mM: NaCl, 85; KCl, 4; Na2B4O7, 15; pH 9.0) was added to the apical side of polarized A6 cells grown on 75-mm filters. Ice-cold amphibian medium was placed basally and plates were overlaid with 35 and 5% sucrose solutions and centrifuged at 190,000 × g for 18 h at 4 °C. Fractions (1 ml) were collected with fraction 1 being at the top of the gradient. Proteins were chloroform/methanol-precipitated, run on SDS-PAGE, and immunoblotted with antibodies to caveolin-1, flotillin, and γ-ENaC. Densitometric quantitation of the immunoblot analysis shown in B. Data are representative of three separate experiments.

**RESULTS**

When A6 cells were extracted with cold Triton X-100 a fraction of all three ENaC subunits appeared in the insoluble pellet (Fig. 1A), suggesting that ENaC might be present in lipid rafts. To determine whether the Triton-insoluble subunits were raft localized we utilized a non-detergent, discontinuous sucrose gradient method for separating membranes of differing buoyant density. When PNS membranes from A6 cells were subjected to discontinuous sucrose density centrifugation (Fig. 1B), it could be shown that a proportion of each subunit was observed in low density fractions 4 and 5 (γ-ENaC is shown) and that flotillin and caveolin, markers of the specific lipid raft subset known as caveolae, also colocalized to these fractions (13). This portion of the gradient corresponds to the interface between 5 and 35% sucrose. Densitometry confirmed that the distribution of γ-ENaC and caveolae between different membrane compartments was similar (Fig. 1C). This finding demonstrated that ENaC subunits are found in the kinds of low density membranes that fit one of the functional definitions of cholesterol-enriched lipid rafts. To further verify that these membranes represented lipid rafts, PNS membranes were treated with 10 mM methyl-β-cyclodextrin (CD) for 1 h at room temperature. CD is a cholesterol-sequestering drug that disrupts the hydrogen bonding interactions between cholesterol and sphingolipids which promote lipid raft formation. Fig. 2 demonstrates that CD treatment results in a significant shift of ENaC subunits to higher density membranes consistent with the loss of cholesterol and disruption of lipid raft structures. Densitometry confirmed that there was a dramatic decrease in low density ENaC and a concomitant increase in channel found at higher density (Fig. 2B). When membranes treated with CD were immunoblotted for caveolin a similar redistribution was observed (bottom two panels of Fig. 2A). Densitometry revealed that the amount of caveolin remaining in raft-like membranes after CD was dramatically reduced.

We next tested whether aldosterone would influence the membrane distribution of ENaC subunits. After 18-h exposure to aldosterone (1 μM), it could be seen that there was a highly reproducible shift in the buoyant density of all three subunits (Fig. 3). γ-ENaC were predominantly located in fraction 4 under control conditions but shifted almost entirely to fraction 5 following aldosterone exposure. For α-ENaC this shift was less dramatic. For all three subunits there was the appearance of a significant proportion of the total subunit pool in higher density fractions (8–12). Interestingly, caveolin was also observed to shift from fraction 4 to fraction 5 upon aldos-
Polarized A6 cells grown on filters were either untreated (−) or were treated with 10 mM cyclodextrin (+) for 1 h at room temperature and then subjected to discontinuous sucrose density centrifugation as described. Fractions (1 ml) were collected with fraction 1 being the top of the gradient. Proteins were chloroform/methanol-precipitated, run on SDS-PAGE, and immunoblotted with antibodies to ENaC subunits or to caveolin. Data are representative of three separate experiments.

**Fig. 2. ENaC Subunits shift from low density membranes to high density membranes upon treatment with cyclodextrin.** A, polarized A6 cells grown on filters were scraped, and a PNS was prepared as described under “Experimental Procedures.” PNSs were left untreated (−) or were treated with 10 mM cyclodextrin (+) for 1 h at room temperature and then subjected to discontinuous sucrose density centrifugation as described. Fractions (1 ml) were collected with fraction 1 being the top of the gradient. Proteins were chloroform/methanol-precipitated, run on SDS-PAGE, and immunoblotted with antibodies to x-ENaC subunits or to caveolin. B, densitometric quantitation of the immunoblot analysis shown to the left in A. Data are representative of three separate experiments.

**Fig. 3. All three ENaC subunits shift to higher density membrane fractions upon chronic exposure to aldosterone.** Polarized A6 cells grown on filters were either untreated (−) or exposed to 1 μM aldosterone for 18 h (+). Following this they were scraped, and a PNS was prepared as described under “Experimental Procedures.” PNSs were left untreated (−) or were treated with 10 mM cyclodextrin (+) for 1 h at room temperature and then subjected to discontinuous sucrose density centrifugation as described. Fractions (1 ml) were collected with fraction 1 being the top of the gradient. Proteins were chloroform/methanol-precipitated, run on SDS-PAGE, and immunoblotted with antibodies to ENaC subunits or to caveolin (Cav). Data are representative of three separate experiments.

Because a large proportion of the ENaC pool is known to reside intracellularly, we wished to examine whether ENaC subunits exist within rafts at the cell surface. Biotinylation of apical cell surface proteins and subsequent isolation using streptavidin beads revealed that ENaC was present in lipid rafts (fractions 4 and 5) in the apical plasma membrane (Fig. 4, PM). Whole cell (WC) lysates were shown for comparison and demonstrate an approximately equivalent distribution between low and high density membranes as can be seen at the cell surface.

**Fig. 4. ENaC is present in lipid rafts at the apical cell surface.** Apical membrane proteins of polarized transporting A6 cells were biotinylated with sulfo-NHS-SS-biotin at 4 °C and then a PNS prepared and run on discontinuous sucrose density gradients as described under “Experimental Procedures.” Fractions removed from the gradient were incubated with streptavidin beads and then the captured biotinylated membranes were pulled down by centrifugation, washed, and immunoblotted as described. PM, apical plasma membrane; WC, whole cell membranes.

**DISCUSSION**

Glycosylphosphatidylinositol-anchored and acylated proteins have been found associated with lipid rafts, and it has been hypothesized that these cholesterol-rich microdomains offer a molecular scaffolding for the concentration of proteins involved in ligand binding and signal transduction as well as in protein sorting at the level of the trans-Golgi network. Proteins that preferentially localize to rafts often have long saturated acyl chains and therefore exhibit a high affinity for liquid-ordered domains (5, 14); however, hydrophobicity alone does not seem to be sufficient for raft localization, as prenylated proteins are excluded (5, 14). As a consequence of these observations, ion channels with multiple membrane-spanning domains were assumed to have little affinity for liquid-ordered membrane domains (5). However, in the last 2 years there have been a limited but growing number of reports demonstrating ion channel association with rafts. These channels include the voltage and Ca2+-activated K+, channel α subunit (hSlo) (15); the voltage-gated K+ channel Kv2.1 (but not Kv4.2), which is found in non-caveolar lipid rafts (16); the voltage-gated K+ channel Kv1.5, which is localized to caveolae (17); the endothelial volume-regulated anion channel (18); and the voltage-gated sodium channel of cardiac myocytes (19), which also localizes to caveolae. The authors in the latter study postulated that observed increases in sodium current in response to β-adrenergic stimulation were occurring as the result of movement of sodium channel out of caveolae and into the sarcolemma. It now appears highly likely that specific ion channels may traffic in, or to, distinct liquid-ordered membrane domains and that their activity might be regulated by these microdomains at the cell surface.

Experiments aimed at defining the membrane microdomain association of ENaC as well as its subcellular distribution are relatively difficult to perform in endogenously expressing cells due to the low abundance of channel protein. As a consequence, a number of groups have used heterologous expression systems that are able to generate higher amounts of protein, as a means to address this question. Prince and Welsh (7) have shown that human ENaC subunits when expressed individually or concurrently in COS-7 or HEK293 cells acquire detergent insolubility at the post-endoplasmic reticulum stage of biosynthesis and that virtually all of the ENaC expressed at the cell surface is detergent-insoluble. These authors speculate that detergent insolubility may be the result of associations with caveolar or cytoskeletal proteins or alternatively be due to self-oligomerization. They did not characterize the buoyant density of their ENaC-containing membranes or attempt to alter the lipid composition of these membranes; therefore a number of interpr-
tations, including lipid raft localization, are possible. In con-
trast, Hanwell et al. (20) expressed rat ENaC in MDCK cells
and showed that ENaC was Triton-soluble and did not float on
Optiprep density gradients, thus demonstrating that ENaC in
this system was not associated with lipid rafts. To investigate
further we turned to A6 cells, which endogenously express
\textit{Xenopus} ENaC and which show appropriate hormone respon-
siveness. Our results clearly demonstrate that a fraction of
both intracellular and plasma membrane ENaC is associated
with lipid rafts by four different biochemical criteria: 1) deter-
genent insolubility, 2) high buoyant density after extraction in 0.5
\text{M Na}_{2}CO_{3}, pH 11.0, 3) colocalization with caveolin and flotillin,
and 4) a dependence on cholesterol for partitioning into high
buoyant density membranes. The reasons for differences in our
findings from those of Hanwell et al. (20) are not clear, but may
reflect the use of a heterologous expression system, overexpression,
or the use of detergent \textit{versus} non-detergent extraction methods
for isolating cellular membranes.

Our results demonstrate that a proportion of all three ENaC
subunits in the \textit{Na}^{+}-transporting A6 epithelium are lipid raft
localized and that this compartmentalization persists at the
plasma membrane. The physiological relevance of such com-
partmentalization is unclear, but the observation that ENaC
subunits can be shifted to membranes of higher density upon
aldosterone treatment suggests strongly that their membrane
domain localization is regulated by appropriate physiological
stims. Indeed, aldosterone is known to alter membrane lipid
association. Further investigation will be required to
understand how lipid rafts influence ENaC trafficking, function,
and turnover.

In conclusion, we have demonstrated that endogenously ex-
pressed ENaC subunits exist within cholesterol-enriched mem-
brane microdomains. Further investigation will be required to
define how lipid rafts influence ENaC trafficking, function,
and turnover.

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Warren G. Hill, Bing An and John P. Johnson

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