Specific Palmitoyltransferases Associate with and Activate the Epithelial Sodium Channel*

Received for publication, January 10, 2017 Published, JBC Papers in Press, January 30, 2017, DOI 10.1074/jbc.M117.776146

Anindit Mukherjee‡, Zhijian Wang‡, Carol L. Kinlough‡, Paul A. Poland‡, Allison L. Marciszyn‡, Nicolas Montalbetti‡, Marcelo D. Carattino§, Michael B. Butterworth‡, Thomas R. Kleymanε, and Rebecca P. Hughey‡

From the Departments of ‡ Medicine, § Cell Biology, and ε Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Edited by Henrik G. Dohlman

The epithelial sodium channel (ENaC) has an important role in regulating extracellular fluid volume and blood pressure, as well as airway surface liquid volume and mucociliary clearance. ENaC is a trimer of three homologous subunits (α, β, and γ). We previously reported that cytoplasmic residues on the β (βCys-43 and βCys-557) and γ (γCys-33 and γCys-41) subunits are palmitoylated. Mutation of Cys that blocked ENaC palmitoylation also reduced channel open probability. Furthermore, toylated ENaC palmitoylation was a dominant role over β subunit palmitoylation in regulating ENaC. To determine which palmitoyltransferases (termed DHHCs) regulate the channel, mouse ENaCs were co-expressed in Xenopus oocytes with each of the 23 mouse DHHCs. ENaC activity was significantly increased by DHHCs 1, 2, 3, 7, and 14. ENaC activation by DHHCs was lost when γ subunit palmitoylation sites were mutated, whereas DHHCs 1, 2, and 14 still activated ENaC lacking β subunit palmitoylation sites. β subunit palmitoylation was increased by ENaC co-expression with DHHC 7. Both wild type ENaC and channels lacking β and γ palmitoylation sites co-immunoprecipitated with the five activating DHHCs, suggesting that ENaC forms a complex with multiple DHHCs. RT-PCR revealed that transcripts for the five activating DHHCs were present in cultured mCCDcl1 cells, and DHHC 3 was expressed in aquaporin 2-positive principal cells of mouse aldosterone-sensitive distal nephron where ENaC is localized. Treatment of polarized mCCDcl1 cells with a γ subunit proteolysis associated with activation were unchanged (20, 21). Mutation of specific Cys palmitoylation sites to Ala reduced channel activity, whereas membrane trafficking and subunit proteolysis associated with activation were unchanged (20, 21). Cell-attached patch clamp analyses of Xenopus oocytes...

ENaCs are amiloride-sensitive Na\(^+\) channels that are found in high resistance epithelia and other tissues. In the aldosterone-sensitive distal nephron (ASDN), ENaC-dependent Na\(^+\) transport has an important role in the maintenance of extracellular fluid volume and blood pressure, as well as extracellular K\(^+\) homeostasis. In the lung, ENaC has a role in regulating airway surface fluid volume, mucociliary clearance, and alveolar fluid volume (1–3). The channels are composed of three homologous subunits (termed α, β, and γ), each with two transmembrane domains, a large extracellular loop and cytoplasmic N and C termini (3–5).

ENaCs are regulated by signaling pathways that modulate its membrane trafficking, residency on the plasma membrane, and degradation (6–8). ENaCs are also regulated by a variety of extracellular factors that affect its open probability (P\textsubscript{o}). These include extracellular cations (H\(^+\), Na\(^+\), and other metals), anions (Cl\(^-\)), laminar shear stress, and proteases that cleave ENaC subunits at specific sites and release embedded inhibitory tracts (1, 2, 9–14). Intracellular phosphorylation, inositol phospholipids, and cytoplasmic Cys palmitoylation also regulate ENaC P\textsubscript{o} (15–21).

Cys palmitoylation of soluble and transmembrane proteins is a reversible post-translational modification that increases protein surface hydrophobicity, facilitating interactions with membranes and/or other proteins (22–26). Although there is no simple consensus sequence for palmitoylation, algorithms predict that more than 50% of human ion channels are palmitoylated (for reviews, see Refs. 22, 26, and 27). Published studies have provided evidence for palmitoylation of numerous voltage-gated channels (e.g. Na\(_{v}\)1.2, Ca\(_{v}\)β2a, and Kv1.1), ligand-gated channels (e.g. NMDA, P\(_2\)X\(_7\), and GABA\(_{A}\)), Ca\(^{2+}\)-activated K\(^+\) channels (e.g. BK), and non-gated channels (e.g. AQP4, connexin, and ENaC) (20, 21, 28–32). The loss of palmitoylation altered channel function by various mechanisms, including effects on channel biogenesis, stability, membrane trafficking, surface expression, and gating.

Using a fatty acid-biotin exchange assay, we found that the β and γ subunits of mouse and human ENaC were Cys palmitoylated, whereas the α subunit was not (20, 21, 33). Sites of cysteine palmitoylation included βCys-43 and βCys-557 in the N and C termini of the mouse β subunit, respectively, as well as γCys-33 and γCys-41 in the N terminus of the γ subunit (20, 21). Mutation of specific Cys palmitoylation sites to Ala reduced channel activity, whereas membrane trafficking and subunit proteolysis associated with activation were unchanged (20, 21). Cell-attached patch clamp analyses of Xenopus oocytes...
expressing channels with subunits lacking selected palmitoylation sites ($\alpha\beta\gamma_{Cys43,41A}, Cys57A_{gamma}, \alpha\beta\gamma_{Cys33A,41A}$) revealed a significantly reduced $P_{Na}$, when compared with wild type ENaC, indicating that palmitoylation affected channel gating (20, 21). Comparison of the activities of channels lacking palmitoylation of one or both subunits ($\alpha\beta\gamma_{Cys43A, Cys57A_{gamma}}, \alpha\beta\gamma_{Cys33A,41A}$, or $\alpha\beta\gamma_{Cys43A, Cys57A_{gamma}, Cys33A,41A}$) showed that $\gamma$ subunit palmitoylation had a dominant role in modulating ENaC activity (21).

Protein palmitoylation is catalyzed by a family of 23 mammalian palmitoyltransferases that exhibit four or more transmembrane domains and a highly conserved cysteine-rich domain adjacent to a DHHC tract (Asp-His-His-Cys) within the active site of the enzyme (34, 35). These palmitoyltransferases, referred to as DHHCs, differ in their relative size and in their cytoplasmic N and C termini, which exhibit protein-interacting motifs such as PDZ-binding domains or ankyrin repeats that likely have roles in subcellular localization and conferring substrate specificity (36, 37). We previously reported that DHHC 2, 3, 7, and 14 but a faint signal for DHHC 1 (Fig. 3, D) showed similar band intensities of the three V5-tagged subunit-specific manner.

Results

Five DHHCs Activate ENaC and Co-immunoprecipitate with the Channel—To identify ENaC-activating DHHCs, the 23 mouse DHHCs with N-terminal HA epitope tags were individually co-expressed with wild type mouse $\alpha\beta\gamma$ ENaC in Xenopus oocytes. Amiloride-sensitive currents were measured after 36–48 h. Five DHHCs (DHHCs 1, 2, 3, 7, and 14) significantly increased ENaC activity ~2-fold (ranging from 1.7 ± 0.9 to 2.5 ± 1.5-fold (means ± S.D.), $p < 0.01$, by one-way ANOVA), whereas the remaining 18 did not significantly alter ENaC activity (Fig. 1A). Because the expression levels of the 23 DHHCs have been variable when expressed in mammalian cells (38, 39), we also assessed their expression in Xenopus oocytes by immunoblotting extracts of oocytes injected with cRNA encoding individual HA-DHHCs. Extracts of oocytes were subjected to immunoblotting with anti-HA antibodies and revealed bands of the expected size for each DHHC, except DHHC 21, although the $\beta$-actin signal by immunoblotting was relatively uniform for all the samples (Fig. 1B). The strongest signals were observed for DHHCs 7, 9, 14, and 25, but of these, only DHHC 7 and 14 were found to activate ENaC when co-expressed. Increased amounts of cRNA were required to obtain signals for DHHCs 4, 8, 11, 13, and 15. Also, longer exposure of the immunoblot to film was needed to obtain signals for DHHCs 4, 6, 11, 20, and 23 (Fig. 1C).

To determine whether ENaC palmitoylation was increased when the channel was co-expressed with an activating DHHC, we transfected HEK293 cells with cDNA for ENaC including a $\beta$ subunit with a C-terminal V5 epitope tag, in the presence or absence of DHHC 7. Palmitoylation of the $\beta$ subunit was assessed with fatty acid exchange chemistry where palmitate is removed from Cys with hydroxylamine treatment, using Tris treatment as a negative control, and replaced with biotin as previously described (20, 21). We observed a significant 2.4-fold increase in $\beta$ subunit palmitoylation when the channel was co-expressed with DHHC 7 (7.7% ± 2.6 (– DHHC 7) versus 18.7% ± 7.4 (+ DHHC 7), means ± S.D., $p < 0.05$) (Fig. 2).

To determine whether the five activating DHHCs associate with ENaC within a protein complex, we co-expressed $\alpha\beta\gamma$ (all subunits with C-terminal V5 tags) and individual DHHCs (DHHC 1, 2, 3, 7, or 14) bearing N-terminal GFP in Fischer rat thyroid (FRT) cells. Cell extracts were immunoprecipitated with anti-V5 antibodies and immunoblotted with anti-GFP antibodies. We found that each of the five DHHCs co-immunoprecipitated with ENaC (Fig. 3, A and B). V5-tagged channels bearing mutations of the four palmitoylation sites ($\alpha\beta\gamma_{Cys43,57A_{gamma}, Cys33,41A}$) also co-immunoprecipitated with the five palmitoyltransferases (Fig. 3, A and B). Based on the intensity of the bands, the most robust co-immunoprecipitating DHHCs were 1 and 7. These results also confirmed our previous findings that DHHC 2 activated and co-immunoprecipitated with ENaC (21). The blots were also reprobed with anti-V5 antibodies and showed similar band intensities of the three V5-tagged ENaC subunits (Fig. 3C), indicating that variations in DHHC co-immunoprecipitation were not due to differential expression or immunoprecipitation of ENaC. Anti-V5 immunoprecipitates from FRT cells expressing the five GFP-DHHCs in the absence of $\alpha\beta\gamma$ENaC revealed no signal on an immunoblot for DHHCs 2, 3, 7, and 14 but a faint signal for DHHC 1 (Fig. 3, D and E). However, the signal for DHHC 1 in the anti-V5 immunoprecipitates was consistently enhanced by co-expression with V5-tagged $\alpha\beta\gamma$ENaC (Fig. 3E). Interestingly, we also found that non-activating DHHC 11 and 23 were also present in anti-V5 immunoprecipitates when co-expressed with V5-tagged $\alpha\beta\gamma$ENaC (Fig. 3, F and G), suggesting that the non-activating DHHCs may also be in complex with the activating DHHCs. Complexes of multiple DHHCs have been previously reported (40).

Palmitoylation of the $\beta$ and $\gamma$ Subunits Are Necessary for Full DHHC-mediated ENaC Activation—We previously reported that channels with mutations that block $\gamma$ subunit palmitoylation (or both $\beta$ and $\gamma$ subunit palmitoylation) are not activated by DHHC 2, whereas channels that lack $\beta$ subunit palmitoylation sites are activated by DHHC 2 (21). These results suggested that there was a degree of subunit specificity regarding channel activation by DHHC 2. We examined whether the other ENaC-activating DHHCs exhibited subunit specificity regarding channel activation. ENaCs lacking palmitoylation sites on either the $\beta$ subunit, the $\gamma$ subunit, or both subunits were expressed in oocytes with or without DHHC 1, 2, 3, 7, or 14. As expected, ENaCs lacking $\beta$ and $\gamma$ subunit palmitoylation sites ($\alpha\beta\gamma_{Cys43,57A_{gamma}, Cys33,41A}$) were not activated by these five DHHCs (Fig. 4A). Furthermore, ENaCs lacking $\gamma$ subunit palmitoylation sites ($\alpha\beta\gamma_{Cys33A,41A}$) were not activated by the five DHHCs (Fig. 4B). However, channels lacking $\beta$ subunit
ENaC activation by specific palmitoyltransferases

Palmitoylation sites (αβC43A,C557Aγ) were significantly activated by DHHCs 1, 2, and 14 but not by DHHCs 3 and 7 (Fig. 4C). These data suggest that ENaC activation by DHHCs 1, 2, and 14 requires Cys palmitoylation of the γ subunit, whereas channel activation by DHHCs 3 and 7 requires Cys palmitoylation of both subunits. These data are also consistent with our earlier results suggesting that γ subunit palmitoylation has a dominant role in activating ENaC (21).

ENaC-activating DHHC 3 is expressed in the ASDN of the Kidney—ENaC is expressed in the latter aspects of the ASDN. Deep sequencing of dissected rat renal tubules indicated that 18 DHHCs are expressed in the ASDN, including the five ENaC-
anti-DHHC 3 antibody was also validated by immunoblot of extracts from FRT cells after transfection with the cDNA for either GFP or GFP-DHHC 3. We observed only a single band in the GFP transfected cells corresponding to the expected size of the endogenous DHHC 3 (M_r = 34 kDa) and a second band in the GFP-DHHC 3 transfected cells corresponding to the expected size of the GFP-tagged protein (62 kDa) (Fig. 5D). We also observed a single band in an immunoblot of kidney homogenates from three individual mice at a size expected for the mouse DHHC 3 (M_r = 34 kDa) that was blocked by antibody preincubation with the antigenic peptide (Fig. 5E).

Palmitoylation Inhibitor 2-Bromopalmitate Blunts Transepithelial Na⁺ Transport in mCCD_cl1 Cells—We examined whether the five ENaC-activating DHHCs were expressed in cultures of a mouse CCD cell line (mCCD_cl1). Using RT-PCR, we identified amplified DNAs representing the expected size for all of the ENaC-activating DHHCs (1, 2, 3, 7, and 14) (Fig. 6). To determine whether palmitoylation has a role in activating endogenous ENaCs in mCCD_cl1 cells, we treated polarized cultures of mCCD_cl1 cells mounted in an Ussing chamber with either 2-bromopalmitate (2-BP), a general irreversible inhibitor of protein palmitoylation, or DMSO (vehicle control) (42–44). We observed a significant decrease in short circuit current (I_sc) within 5 min after apical addition of 2-BP that reached a new steady state I_sc after 30 min (Fig. 7, A and B). The amiloride-sensitive I_sc was 46.2 ± 7.2 μA/cm² (n = 9) in vehicle-treated cells and 10.1 ± 3.2 μA/cm² (n = 9) in 2-BP-treated cells (p < 0.001, by one-way ANOVA followed by a Tukey post hoc test), 2-BP treatment resulted in a significant decrease in transepithelial resistance (TER) after 30 min (pretreatment, 15.8 ± 2.4 kΩ × cm² (n = 5, mean ± S.D.) versus post treatment, 2.9 ± 0.9 kΩ × cm² (n = 5), p < 0.01, by one-way ANOVA followed by a Tukey post hoc test), whereas vehicle treatment increased resistance after 30 min (pretreatment, 17.2 ± 5.4 kΩ × cm² (n = 6) versus post-treatment 21.9 ± 7.9 kΩ × cm² (n = 6), p < 0.01, by one-way ANOVA followed by a Tukey post hoc test), suggesting that 2-BP is modifying tight junctions (Fig. 7, A and C). Apical treatment of mCCD_cl1 cells with amphotericin B after amiloride washout acutely increased I_sc in both vehicle-treated and 2-BP-treated cells (peak current was 87.6 ± 27.4 μA/cm² for vehicle control (n = 6) and 37.5 ± 9.5 μA/cm² for 2-BP-treated (n = 5), p < 0.01, by unpaired one-way ANOVA followed by a Tukey post hoc test), indicating that the 2-BP cells were viable (Fig. 7A). Steady state I_sc following amphotericin addition for vehicle-treated and 2-BP-treated cells were similar (38.3 ± 10.8 μA/cm² for vehicle-treated versus 34.6 ± 9.8 μA/cm² for 2-BP treated, (n = 6), p > 0.05 by unpaired Student’s t test).

Discussion

Results from numerous studies indicate that ~400 proteins in mammalian cells and tissues are palmitoylated. The identification of palmitoylated proteins has been a slow process. The first acylated proteins were described in 1979, whereas the first global analysis of palmitoylated yeast proteins and palmitoylated rat brain synaptosomal proteins were performed in 2006 and 2008, respectively (45–48). Palmitoylation was established by a variety of increasingly complex methodologies starting...
FIGURE 3. ENaC co-immunoprecipitates with the five activating DHHCs. FRT cells were transfected with WT $\alpha \beta \gamma$ ENaC or ENaC with mutant subunits lacking sites for palmitoylation ($\alpha \beta$Cys43,557A; Cys33,41A) either alone (−) or with DHHC 1, 2, 3, 7, or 14 as indicated. All DHHCs had an N-terminal GFP epitope-tag and all three ENaC subunits had a C-terminal V5 epitope tag. A, an aliquot of the cell extract (10% total extract) was retained for immunoblotting (IB) with anti-GFP antibodies. B, the remainder was incubated with anti-V5 antibodies. Total extract and IPs were subjected to immunoblotting with anti-GFP antibodies. C, immunoblots of the IP were stripped and probed with anti-V5 antibodies to assess expression of $\alpha \beta \gamma$ ENaC subunits. Mobility of Bio-Rad Precision Plus protein standards is indicated on the right of each blot. The co-immunoprecipitating DHHCs are noted in B with an arrowhead and correspond to the predicted Mr for GFP-tagged DHHC1 (77,978), DHHC2 (66,981), DHHC3 (59,041), DHHC7 (60,213), and DHHC14 (78,658). A nonspecific (ns) band was present in all lanes as indicated in B. The results are representative of three independent experiments.

D and E, FRT cells expressing GFP-tagged DHHC 1, 2, 3, 7, and 14 were expressed in the absence of ENaC, whereas ENaC with three V5-tagged subunits was expressed alone (−) or with DHHC 1. Total cell extract (D) and anti-V5 IPs (E) were subjected to immunoblotting with anti-GFP antibodies. Note that the signal for DHHC1 in the IP was greatly enhanced by co-expression of ENaC (E). F and G, ENaC with a V5-tagged $\alpha$ subunit was co-expressed in FRT cells alone (−), with an HA-tagged activating DHHC (DHHC 7), or with an HA-tagged non-activating DHHC (DHHC 11 or 23). Cell extracts (F) and anti-V5 IPs (G) were immunoblotted with anti-HA antibodies conjugated to HRP. The results are representative of three independent experiments.
ENaC Activation by Specific Palmitoyltransferases

with radiolabeling with [3H]palmitate and moving on to acyl-biotin exchange protocols, acyl-resin-assisted capture with thiopeptyl-Sepharose and click chemistry based on metabolic labeling with bioorthogonal palmitate analogs combined with quantitative mass spectroscopy (45, 46, 49, 50).

ENaCs are among the ~50 ion channels known to be palmitoylated (for reviews, see Refs. 22, 26, and 51). We treated mCCDcl1 cells that express endogenous ENaC with an inhibitor of protein acylation (2-BP) and found a rapid reduction in ENaC currents consistent with the reduced activity of ENaC that we observed in heterologous systems when we expressed mutant ENaC lacking sites for Cys palmitoylation (20, 21). However, the reduced currents could also reflect changes in palmitoylation of additional proteins that either alter ENaC trafficking or gating. The palmitate analog 2-BP has been regularly used in cultured cells to inhibit palmitoylation of proteins, thereby providing a complementary in vivo approach to study the function of palmitate addition to specified proteins (44, 52). Although 2-BP clearly inhibits the palmitoyltransferases, there is evidence that it inhibits the thioesterases as well (44, 52, 53). 2-BP also inhibits metabolic enzymes, including fatty acid CoA ligase, which reduces the cellular content of the palmitoylation substrate palmitoyl-CoA (44, 52, 53). We also observed a decrease in TER after treatment of mCCDcl1 cells with 2-BP, which may reflect reduced palmitoylation of claudins normally found within the tight junctions (54–56). Transcripts for 13 claudins were identified in dissected rat kidney CCDs by deep sequencing (41). A role of claudin palmitoylation in modifying TER was noted in studies of polarized Madin-Darby canine kidney cells, where TER was increased 5-fold after transfection with wild type claudin-14. However, TER was unchanged after transfection with claudin-14 lacking sites for palmitoylation because of reduced targeting to the tight junctions (55).

Of the 23 mouse palmitoyltransferases, DHHCs 1, 2, 3, 7, and 14 activated ENaCs expressed in Xenopus oocytes. Analysis of gene expression data in the online Xenbase indicates that DHHCs 4, 6, 8, 14, and 16 are expressed in Xenopus laevis oocytes, whereas DHHCs 1, 2, 3, 7, 9, 15, 20, and 21 are not expressed. There are no data available for DHHCs 5, 11, 12, 13, 17, 23, 24, and 25. Although these data indicate that oocytes do express one ENaC-activating transferase (DHHC 14), overexpression of DHHC 14 further enhanced ENaC activity. These five activating DHHCs are expressed in the ASDN (41). ENaCs are expressed in principal cells in the latter aspect of the ASDN, and we found transcripts for all five ENaC-activating DHHCs in a cultured principal cell line (mCCDcl1) using RT-PCR. Furthermore, in mouse kidney sections DHHC 3 was localized in cells expressing aquaporin 2, a principal cell marker. At least three of the channel activating DHHCs (DHHCs 1, 2, and 3) are expressed in mouse airway epithelia (57), whereas analyses of DHHC expression in both human kidney and lung by RT-PCR revealed expression of 17 DHHCs (including DHHCs 1, 3, 7, and 14) (58). Taken together, these data are consistent with a role for Cys palmitoylation in regulation of ENaC in both kidney and lung, because the relevant transferases are expressed at the appropriate sites in these tissues.

Although the list of palmitoylated proteins continues to expand, it has been a challenge to identify physiologically relevant DHHC-substrate pairs as (i) there are numerous reports indicating that substrates are shared by multiple DHHCs, (ii) tissue and cell type expression of the DHHCs are highly variable, and (iii) the definitive subcellular distribution of each DHHC has been described in only a few cases (for review, see Refs. 26 and 27). Expression of epitope-tagged DHHCs in HEK293 cells coupled with immunofluorescence microscopy placed DHHCs 1, 2, 3, 6, 10, 11, 12, 13, 14, 16, 19, and 22 in the

FIGURE 4. ENaC-activating DHHCs exhibit β and γ subunit specificity. Xenopus oocytes were injected with cRNAs for wild type αβγ or mutant ENaCs lacking sites for palmitoylation on both subunits (αβγCys43,557AγCys33,41A) (A), the γ subunit (αβγC33A,C41A) (B), or the β subunit (αβC43A,G557Aγ) (C). Mutant ENaCs were expressed alone (NA, no addition, n = 77–98) or co-expressed with DHHCs 1, 2, 3, 7, or 14 as indicated (n = 11–33). Amiloride-sensitive currents were measured 48 h after cRNA injection and normalized to wild type αβγ currents each day. The data are presented as box and whisker plots, with wild type αβγ set as 1 (dashed line, n = 73–115). Co-expression of DHHCs with ENaC lacking palmitoylation sites on both the β and γ subunit (A) or just the γ subunit (B) did not affect channel activity (p > 0.05 versus NA). C, co-expression of DHHCs 1, 2, or 14 with ENaC lacking β subunit palmitoylation sites significantly activated the channel (gray boxes) (p < 0.01 for DHHC 1 or 2 versus NA, p < 0.05 for DHHC 14 versus NA, determined with one-way ANOVA followed by a Tukey test). Whiskers indicate the 10th and 90th percentiles. The median is indicated by a horizontal line, and the mean is indicated with a cross symbol within each bar.
endoplasmic reticulum (ER); DHHCs 2, 3, 4, 7, 8, 12, 15, 17, 18, and 22 in the Golgi apparatus; and DHHCs 5, 20, and 21 at the plasma membrane (58). Recent studies have confirmed DHHC 6 expression in the ER and DHHC 3 expression in the Golgi apparatus, whereas DHHCs 1, 2, and 11 were found in endosomes (41), and DHHCs 5 and 8 were found at the plasma membrane (reviewed in Ref. 27). It is now clear that DHHCs 4 and 6 are localized to the ER by conserved C-terminal lysine-based sorting signals (e.g. \( K \text{XXX} \)) (34). Not surprisingly, there are now several examples whereby subcellular localization of DHHC substrates (e.g. H/N-Ras and small G proteins \( \text{G}^{\text{H9251}} \)) are regulated by palmitoylation-depalmitoylation cycles based on the localization of DHHCs and putative depalmitoylating enzymes such as acyl-protein thioesterase 1 (59, 60).

In summary, we have identified five ENaC-activating DHHCs and found that ENaC was present in a stable complex (or complexes) with DHHCs 1, 2, 3, 7, and 14. Additional non-activating DHHCs such as DHHC 11 and 23 are likely present in the complex (or complexes) because they were also found in ENaC immunoprecipitates when co-expressed in FRT cells. Complexes of heterogeneous DHHCs have been previously reported (40). It is also possible that ENaC could be activated by

**FIGURE 5.** ENaC-activating DHHC 3 is expressed in mouse kidney ASDN. A, mouse kidneys were fixed in PFA, and cryosections were incubated with goat anti-aquaporin 2 antibodies as a marker of principal cells in the ASDN and rabbit anti-DHHC 3 antibodies, followed by a FITC-tagged anti-goat antibody (green) and a Cy3-tagged anti-rabbit antibody (red). Nuclei were counterstained with TO-PRO 3 (blue). B, preincubation of the anti-DHHC 3 antibody with the antigenic peptide selectively prevented staining (red) without interfering with staining for aquaporin 2 (green). Slides were imaged by confocal microscopy as described under “Experimental Procedures.” The white bar in the merged images is 10 \( \mu \text{m} \). The results are representative of three independent experiments. C, the yellow box in the Merge panel in A is enlarged to emphasize the subapical intracellular staining for DHHC3 in principal cells with apical aquaporin 2 staining. Further validation of the anti-DHHC3 antibody is shown in D and E. D, FRT cells were transfected with either GFP or GFP-DHHC3, and cell extracts were immunoblotted (IB) with anti-DHHC3 antibodies. Bands for the endogenous DHHC3 (34 kDa) and the transfected GFP-DHHC3 (62 kDa) are indicated to the right of the panel. E, kidneys from three different mice were homogenized, and duplicate aliquots were immunoblotted with anti-DHHC3 antibody preincubated with or without the antigenic peptide. D and E, blots were stripped and probed with anti-\( \beta \)-actin antibodies as a loading control. The mobility of the Bio-Rad Precision Plus protein standards is shown to the left of each panel.

**FIGURE 6.** Transcripts for ENaC-activating DHHCs are expressed in mCCDcl1 cells. Single-stranded cDNA was generated from total RNA isolated from cultures of mCCDcl1 cells using reverse transcriptase (+ RT) and amplified using PCR with primer pairs specific for mouse DHHC 1, 2, 3, 7, or 14 that span at least one intron. The reactions without RT (−) were analyzed with PCR as a negative control. An aliquot of each PCR was analyzed on a 1% agarose gel as indicated and corresponded to the expected size of the amplified fragments (474 bp for DHHC1, 475 bp for DHHC2, 453 bp for DHHC3, 541 bp for DHHC7, and 420 bp for DHHC 14). A nonspecific band in the − RT lane for DHHC2 is noted by (>) and corresponds to the expected size of a product overlapping an intron. Mobility of lambda (λ) DNA digested with HindIII (562 bp) and low DNA mass ladder markers (LM, 200, 400, 800, and 1600 bp) are indicated on the left of the gel as standards (std, from Invitrogen). The results are representative of three independent experiments.
FIGURE 7. ENaC activity in mCCD<sub>cl1</sub> cells is reduced by an inhibitor of palmitoylation. A, polarized cultures of mCCD<sub>cl1</sub> cells growing on permeable supports were placed in a Ussing chamber. $I_{sc}$ was monitored before and for 30 min after apical addition of DMSO (vehicle (1:2,000 dilution), top profile) or 25 μM 2-BP (bottom profile). The ENaC-dependent components of the $I_{sc}$ were determined by the addition of apical amiloride (Amil, 10 μM). After washing out amiloride, the integrity of the epithelium was assessed by apical addition of amphotericin B (120 μg/ml). B, amiloride-sensitive $I_{sc}$ ($I_{amil}$) prior to addition of DMSO or 2-BP (control) was compared with that of DMSO-treated (vehicle) and 2-BP-treated cells. The experiment was carried out 5–9 times, and the data are presented as box and whisker plots (gray box, $p < 0.001$ versus control, by one-way ANOVA). C, TER of pretreated cells of each group (Pre) was compared with that of cells at the end of 30 min treatment (Post) with either DMSO (vehicle) or 2-BP (gray boxes). $p < 0.01$, one-way ANOVA versus pretreatment control. Whiskers indicate the 10th and 90th percentiles. Median is indicated by a horizontal line, and the mean is indicated with a cross symbol within each box.
ENaC Activation by Specific Palmitoyltransferases

additional DHHCs in other cell types where unknown factors stabilize DHHC complexes. Because channel activating DHHCs are likely expressed at different sites in the biosynthetic pathway, including ER (for DHHCs 1, 2, 3, and 14), Golgi (DHHCs 2, 3, and 7) and endosomes (DHHCs 1 and 2), it is likely that ENaC is palmitoylated during transit through the biosynthetic pathway and during endocytic recycling at the apical cell surface.

Experimental Procedures

Plasmids—cDNAs for wild type mouse α, β, and γ ENaC subunits and mutant subunits (βC43A,C557A and γC33A, C41A), with and without N-terminal HA and C-terminal V5 epitope tags, or an α subunit with only a C-terminal V5 tag were described previously (12, 20, 21). The 23 cDNAs encoding individual mouse palmitoyltransferases with an N-terminal HA epitope tag (in pEF-Bos-HA) and with an N-terminal GFP tag (in pEGFP-C1) were a gift from Masaki Fukata (National Institute for Physiological Sciences, Okazaki, Japan) and were previously described (35). Each HA-tagged DHHC cDNA was subcloned into pCDNA 3.1(−)neo, and corresponding cRNAs for oocyte injections were prepared using T7 mMESSAGE mMACHINE® kit (Ambion Invitrogen).

Cell Culture and Co-immunoprecipitations—The mCCDcl1 cells (kindly provided by B. Rossier and L. Schild, Université de Lausanne, Lausanne, Switzerland) were grown in 10-cm-diameter cell culture dishes (passages 30–40) in defined growth medium (DMEM supplemented with insulin (5 μg/ml), human apotransferrin (5 μg/ml), EGF (10 ng/ml), T3 (1 mM), dexamethasone (50 nM), sodium selenite (0.06 nM), penicillin (100 μg/ml), streptomycin (130 μg/ml), and 2% decomplemented FCS at 37 °C in 5% CO2 as described previously (61–63). The medium was changed every 2–3 days. For all electrophysiology experiments, the mCCDcl1 cells were plated on permeable Snapwell filter supports for 3–5 days (0.4-m pore size, 1.12-cm² surface area; Corning, Lowell, MA). Formation of tight junctions by mCCDcl1 cells were verified by measuring TER before electrophysiological analysis in a Ussing chamber.

FRT cells were provided by P. Snyder (University of Iowa) and cultured in DMEM/F-12 medium supplemented with 7.5% fetal bovine serum as previously described (64). The cells were transfected with cDNAs using Lipofectamine 2000 (Invitrogen Thermo Fisher) according to the manufacturer. FRT cells were transfected with 0.5 μg/subunit for ENaC and 0.5 μg for each GFP-tagged DHHC. For co-immunoprecipitation experiments, FRT cells were transfected with N-terminal HA and C-terminal V5 tagged WT αβγ or ENaC with mutant subunits lacking sites for palmitoylation (αβCys43,557AγCys33,41A) either alone or with N-terminal GFP tagged DHHC 1, 2, 3, 7, or 14 (cDNAs described below). The following day, detergent extracts of the cells were incubated with agarose-immobilized goat anti-V5 antibodies (Bethyl Laboratories, Inc., Montgomery, TX), and immunoprecipitates were subjected to SDS-PAGE and immunoblotting with either rabbit anti-GFP antibody (2 μg/ml; Thermo Fisher Molecular Probes) or mouse monoclonal anti-V5 antibodies (at 1 μg/ml; Bio-Rad) as previously described (21). 10% of the detergent cell extracts was subjected to SDS-PAGE and immunoblotting with a rabbit anti-GFP antibody (2 μg/ml). Alternatively, co-immunoprecipitation experiments were carried out with HA-DHHCs 7, 11, or 23 and ENaC with non-tagged β and γ subunits and a C-terminal V5 epitope tagged α subunit. Anti-V5 immunoprecipitates or cell extracts were immunoblotted with rat monoclonal anti-HA antibodies conjugated to HRP at 0.05 μg/ml prepared as directed by the manufacturer (Roche Diagnostics).

Assay for Cys Palmitoylation in HEK293 Cells—HEK293T cells (ATCC Cell Biology Collection) were cultured in DMEM/F-12 medium supplemented with 8% fetal bovine serum. The cells were plated in a 12-well size dish and transfected the next day with αβγ ENaC (β subunit with a C-terminal V5 epitope tag and non-tagged α and γ subunits), with or without DHHC 7 (with an N-terminal HA tag) using Lipofectamine 2000 according to the manufacturer. Cys palmitoylation of the β subunit in anti-V5 immunoprecipitates (IPs) was assessed with fatty acid exchange chemistry where palmitate is removed from Cys with hydroxylamine treatment, using Tris treatment as a negative control, and replaced with biotin as previously described (21). A fraction of the IP (10%) was reserved to assess total β subunit in the initial immunoprecipitate, and biotinylated β subunit was recovered with avidin-conjugated beads (90%) for immunoblotting with mouse anti-V5 antibodies at 4 μg/ml (Invitrogen; R96025). The percentage of palmitoylation was calculated from the difference in β subunit biotinylation after treatment with hydroxylamine or Tris, relative to the β subunit recovered in the total immunoprecipitate.

Functional ENaC Expression in Xenopus Oocytes—Two-electrode voltage clamp was performed as previously described (65, 66). Oocytes were injected with wild type or mutant α, β, and γ mouse ENaC subunit cRNAs (0.5–1 ng/subunit) and co-injected where noted with cRNAs for one of the 23 mouse DHHCs (3 ng). The oocytes were incubated at 18 °C in modified Barth’s saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 15 mM HEPES, 10 μg/ml streptomycin sulfate, 100 μg/ml gentamicin sulfate, pH 7.4) for 36–48 h before electrophysiological recordings were performed at room temperature. Oocytes were placed in a recording chamber and perfused with a solution containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl2, 10 mM HEPES, pH 7.4. Inward Na+ currents were measured at −100 mV in the absence and presence of amiloride (10 μM). The protocol for harvesting oocytes from X. laevis was approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunoblotting of DHHCs Expressed in Xenopus Oocytes—20 oocytes were injected with cRNAs (12 ng) for 1 of the 23 mouse DHHCs containing an N-terminal HA epitope tag. After 48 h, the oocytes were extracted in detergent for immunoblotting as described previously (20, 21). Briefly, surviving oocytes (n = 5–17) were disrupted in homogenization buffer (100 mM NaCl, 50 mM Tris HCl, pH 7.4) including 1% protease inhibitor mixture III (EMD Millipore, Billerica, MA) using an allgoy syringe (1 cc/ml, 27-gauge × 0.5-inch needle; Terumo Medical Corporation, Elkton, MD) in a 1.5-ml capped tube (15 μl buffer/oocyte). The homogenates were centrifuged twice at 200 × g for 10 min, and the supernatant was removed from the pellet of yolk proteins, nuclei, and cell debris to a clean tube. Each supernatant was adjusted to 1% Triton X-100 by addition of 1/3 vol
volume of 4% Triton X-100 in homogenization buffer including protease inhibitors and incubated overnight on a rotating wheel at 4 °C before centrifugation at 15,300 × g at 4 °C to remove any insoluble material. An aliquot equivalent to 1.2 oocytes was subjected to immunoblotting with mouse anti-β-actin ascites (A5441 clone AC-15, diluted 1:1000; Sigma-Aldrich) or rat anti-HA antibodies conjugated to HRP (0.05 μg/ml; Roche Diagnostics). Signal for some DHHCs on immunoblots was observed only after increasing the amount of cRNA injected into oocytes (388 ng for DHHC 4, 46 ng for DHHC 8, 67 ng for DHHC 11, 79 ng for DHHC 13, and 67 ng for DHHC 23). No signal for DHHC 21 was observed after injection of 20 ng.

RNA Isolation and RT-PCR—Total RNA was isolated from three 10-cm diameter cell culture dishes of mCCD16i cells using a PureLink RNA mini kit (Ambion). Single-stranded cDNA was generated from 1 μg of total RNA using the Superscript III (ThermoFisher) kit. The cDNA was amplified by PCR using primers designed against nucleotide sequences that are invariant between Xenopus, rat, mouse, and human ENaC-activating DHHCs: DHHC1 forward, CACGGATGTGTGGTTTGT-GTT; DHHC1 reverse, AGACCTGGCACTCATACACTC; DHHC2 forward, GAAAGGATGATCATGTT; DHHC2 reverse, CACTGTGATTAGTCCC; DHHC3 forward, AAGCGGTGTCAGCAAG; DHHC3 reverse CATACT-GGTACGGGTCTG; DHHC7 forward, GCCACGAAGGAGTCGCAAG; DHHC7 reverse, CTAGTGTGAGAAAGCAGC; DHHC14 forward GACAGAAGAGCTATGTCAG; and DHHC14 reverse, GCATGTGAGAACGTATG. Reactions were electrophoresed on a 1% agarose gel with low DNA mass ladder markers (Invitrogen) and visualized using GelReD stain (Biotium Inc., Hayward, CA) in a Bio-Rad DNA mass ladder markers (Invitrogen) and visualized using GelReD stain (Biotium Inc., Hayward, CA) in a Bio-Rad molecular imager GelDoc XR* imaging system. Expected PCR-amplified fragment sizes for DHHCs 1, 2, 3, 7, and 14 are 474, 475, 453, 541, and 420 bp, respectively.

Immunostaining and Imaging of Mouse Kidneys—The protocol for harvesting kidneys from mice was approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Kidneys from C57BL/6 male mice were fixed in 4% PFA for 16 h. Kidneys from C57BL/6 male mice were fixed in 4% PFA for 16 h. The kidneys were washed in PBS, and cryosections of 0.5 μm thick were stained with primary antibodies (rabbit anti-DHHC 3 antibody (1 μg/ml, ab31882; Abcam), or mouse anti-DHHC 3 antibody (A5441 clone AC-15, diluted 1:1000; Sigma-Aldrich) as a loading control. Signal for DHHC 3 on immunoblots was detected using either BioMax MR film (Carestream Health, Inc., Rochester, NY) or a Bio-Rad imaging system.


ting. Average TER was calculated from five bipolar pulses of 10 mV prior to addition of DMSO or 2-BP and five pulses prior to addition of amiloride. After 5 min, amiloride was washed out, and the Isc was allowed to stabilize again. The apical membrane


d volume of anti-aquaporin 2 antibody (0.4 μg/ml) before primary antibodies were applied to tissue.

The anti-DHHC 3 antibody was further validated by immunoblotting. FRT cells were transfected with the cDNA for either GFP or DHHC 3 with an N-terminal GFP epitope tag using Lipofectamine 2000 (Invitrogen Thermo Fisher) according to the manufacturer. Cells were extracted in detergent, and an aliquot was subjected to immunoblotting with either mouse anti-β-actin ascites (A5441 clone AC-15, diluted 1:1000; Sigma-Aldrich) or rabbit anti-DHHC 3 antibody (1 μg/ml, ab31837; Abcam). Kidneys from three C57BL/6 mice were homogenized separately, and detergent extracts (30 μg) of the post-nuclear supernatant were subjected to immunoblotting on two identical blots with rabbit anti-DHHC 3 antibody (1 μg/ml, ab31837; Abcam) preincubiated with and without the immunizing peptide in 10-fold excess (0.75 μg of antibody with 7.5 μg of peptide, ab31882;Abcam). Both blots were also immunoblotted with mouse anti-β-actin ascites (A5441 clone AC-15, diluted 1:1000; Sigma-Aldrich) as a loading control. Signal for DHHC 3 on immunoblots was detected using either BioMax MR film (Carestream Health, Inc., Rochester, NY) or a Bio-Rad ChemiDoc Touch imaging system.

Isc, Measurements—mCCD16i cells were grown on Snapwell inserts (Corning Costar) until a confluent, high resistance monolayer was obtained. Monolayer resistances were greater than 1 kΩ × cm² for all cell monolayers tested. Snapwells were mounted in Ussing sliders (P2302; Physiological Instruments, San Diego, CA) and inserted into the chambers of an EM-CSYS Ussing system (Physiologic Instruments) equipped with a heat block for temperature control. The apical and basolateral hemichambers contained 4 ml of Krebs buffer solution (110 mM NaCl, 25 mM NaHCO₃, 5.8 mM KCl, 2 mM MgSO₄, 1.2 mMKH₂PO₄, 2 mM CaCl₂, and 11 mM glucose). The chamber temperature was maintained at 37 °C. The hemichambers were continuously bubbled with 95% O₂, 5% CO₂, which maintained the pH at 7.4. The apical and basolateral hemichambers were connected to Ag/AgCl electrodes via 5 mM NaCl agar bridges for voltage sensing and current passing. The electrodes were connected to a VCC MC6 multichannel voltage/current clamp (Physiologic Instruments). The asymmetry of voltage-sensing Ag/AgCl electrodes and the liquid junction potentials were compensated using an offset removal circuit before tissue mounting. Isc and TER were measured under voltage clamp conditions. To calculate TER, a bipolar pulse of ± 10 mV with a duration of 0.5 s was applied every 60 s. The data were digitized at 1 kHz using DigiData 1440A (Molecular Devices) and acquired with pClamp 10.3 software (Molecular Devices). After an equilibration period to achieve a stable Isc (~30 min), the cells were treated with either 25 μM 2-BP (2-bromohexadecanoic acid (97% pure); Aldrich) or vehicle only (DMSO (1:2,000 dilution) in the apical hemi-chamber for 30 min. The amiloride-sensitive component of the Isc was then determined by adding 10 μM amiloride to the apical hemi-chamber. Typically, ≥90% of the total Isc was inhibited after amiloride addition. Average TER was calculated from five bipolar pulses of ± 10 mV prior to addition of DMSO or 2-BP and five pulses prior to addition of amiloride. After 5 min, amiloride was washed out, and the Isc was allowed to stabilize again.
ENaC Activation by Specific Palmitoyltransferases

was then permeabilized by the addition of amphotericin B (120 μg/ml; Sigma).

Data and Statistical Analyses—The data are expressed as the means ± S.D. Box and whisker plots are used in figures to show the distribution of data: median (middle line); mean (cross or dot); 25th to 75th percentile (box); and 10th and 90th percentiles (whisker). Experiments were repeated with a minimum of two batches of oocytes obtained from different frogs. When appropriate, statistical comparisons were obtained from unpaired Student’s t test or one-way ANOVA followed by a Tukey post hoc test. A p value of less than 0.05 was considered statistically different.

Author Contributions—T. R. K. and R. P. H. conceived and designed the study and wrote the paper. A. M. carried out studies in FRT cells and wrote the paper. A. M. and Z. W. carried out studies in Xenopus oocytes. A. M., N. M., M. B. B., and M. D. C. carried out studies with mCCDcl1 cells in Ussing chambers. A. L. M. prepared the mouse kidney slices and carried out the confocal microscopy. Z. W. and P. A. P. subcloned the 23 DHHCs into new vectors. C. L. K. carried out the fatty acid exchange chemistry assay for ENaC palmitoylation.

Acknowledgments—Plasmids encoding the 23 DHHCs were a gift from Masaki Fukata (National Institute for Physiological Sciences, Okazaki, Japan). The mCCDcl1 cells were kindly provided by B. Rossier and L. Schild (Univerité de Lausanne, Lausanne, Switzerland). Antibodies for immunostaining were provided by the lab of Gerard Apodaca (University of Pittsburgh) and the Pittsburgh Center for Kidney Research.

References
1. Rossier, B. C., and Stutts, M. J. (2009) Activation of the epithelial sodium channel (ENaC) by serine proteases. Annu. Rev. Physiol. 71, 361–379
2. K영상han, O. B., and Kleyman, T. R. (2012) Epithelial Na⁺ channel regulation by cytoplasmic and extracellular factors. Exp. Cell Res. 318, 1011–1019
3. Kellenberger, S., and Schild, L. (2015) International Union of Basic and Clinical Pharmacology: XCL structure, function, and pharmacology of acid-sensing ion channels and the epithelial Na⁺ channel. Pharmacol. Rev. 67, 1–35
4. Canessa, C. M., Merillat, A. M., and Rossier, B. C. (1994) Membrane topology of the epithelial sodium channel in intact cells. Am. J. Physiol. 267, C1682–C1690
5. Kashlan, O. B., and Kleyman, T. R. (2011) ENaC structure and function in the wake of a resolved structure of a family member. Am. J. Physiol. Renal Physiol. 301, F684–F696
6. Bhal, V., and Hallows, K. R. (2008) Mechanisms of ENaC regulation and clinical implications. J. Am. Soc. Nephrol. 19, 1845–1854
7. Eaton, D. C., Malik, B., Bao, H. F., Yu, L., and Jain, L. (2010) Regulation of epithelial sodium channel trafficking by ubiquitination. Proc. Am. Thorac. Soc. 7, 54–64
8. Ronzaud, C., and Staub, O. (2014) Ubiquitylation and control of renal Na⁺ balance and blood pressure. Physiology (Bethesda) 29, 16–26
9. Sheng, S., Bruns, J. B., and Kleyman, T. R. (2004) Extracellular histidine residues crucial for Na⁺ self-inhibition of epithelial Na⁺ channels. J. Biol. Chem. 279, 9743–9749
10. Collier, D. M., and Snyder, P. M. (2009) Extracellular protons regulate human ENaC by modulating Na⁺ self-inhibition. J. Biol. Chem. 284, 792–798
11. Collier, D. M., and Snyder, P. M. (2009) Extracellular chloride regulates the epithelial sodium channel. J. Biol. Chem. 284, 29320–29325
12. Hughay, R. P., Mueller, G. M., Bruns, J. B., Kinlough, C. L., Poland, P. A., Harkleroad, K. L., Carattino, M. D., and Kleyman, T. R. (2003) Maturation of the epithelial Na⁺ channel involves proteolytic processing of the α- and γ-subunits. J. Biol. Chem. 278, 37073–37082
13. Hughay, R. P., Bruns, J. B., Kinlough, C. L., Harkleroad, K. L., Tong, Q., Carattino, M. D., Johnson, J. P., Stockand, J. D., and Kleyman, T. R. (2004) Epithelial sodium channels are activated by furin-dependent proteolysis. J. Biol. Chem. 279, 18111–18114
14. Ergonul, Z., Friindt, G., and Palmer, L. G. (2006) Regulation of maturation and processing of ENaC subunits in the rat kidney. Am. J. Physiol. Renal Physiol. 291, F683–F693
15. Ma, H. P., Chou, C. F., Wei, S. P., and Eaton, D. C. (2007) Regulation of the epithelial sodium channel by phosphatidylinositides: experiments, implications, and speculations. Pflugers Arch. 455, 169–180
16. Yang, L. M., Rinke, R., and Korbmacher, C. (2006) Stimulation of the epithelial sodium channel (ENaC) by cAMP involves putative ERK phosphorylation sites in the C termini of the channel’s β- and γ-subunit. J. Biol. Chem. 281, 9859–9866
17. Pochynuk, O., Tong, Q., Medina, I., Vandewalle, A., Staruschenko, A., Bugay, J., and Stockand, J. D. (2007) Molecular determinants of PI(4,5)P2 and PI(3,4,5)P3 regulation of the epithelial Na⁺ channel. J. Gen. Physiol. 130, 399–413
18. Pochynuk, O., Tong, Q., Staruschenko, A., Ma, H. P., and Stockand, J. D. (2006) Regulation of the epithelial Na⁺ channel (ENaC) by phosphatidylinositides. Am. J. Physiol. Renal Physiol. 290, F949–F957
19. Pochynuk, O., Bugay, J., and Stockand, J. D. (2008) Physiologic regulation of the epithelial sodium channel by phosphatidylinositides. Curr. Opin. Nephrol. Hypertens. 17, 533–540
20. Mueller, G. M., Maarouf, A. B., Kinlough, C. L., Sheng, N., Kashlan, O. B., Okumura, S., Lutty, S., Kleyman, T. R., and Hughay, R. P. (2010) Cys palmitoylation of the β subunit modules gating of the epithelial sodium channel. J. Biol. Chem. 285, 30453–30462
21. Mukherjee, A., Mueller, G. M., Kinlough, C. L., Sheng, N., Wang, Z., Mutyfata, S. A., Kashlan, O. B., Kleyman, T. R., and Hughay, R. P. (2014) Cys palmitoylation of the γ subunit has a dominant role in modulating activity of the epithelial sodium channel. J. Biol. Chem. 289, 14351–14359
22. Shipston, M. J. (2011) Ion channel regulation by protein palmitoylation. J. Biol. Chem. 286, 8709–8716
23. Nadaslki, M. J., and Lindner, M. E. (2007) Protein lipidation. FEBS J. 274, 5202–5210
24. Lindner, M. E., and Deschenes, R. J. (2007) Palmitoylation: policing protein stability and traffic. Nat. Rev. Mol. Cell Biol. 8, 74–84
25. Yeste-Velasco, M., Lindner, M. E., and Lu, Y. J. (2015) Protein S-palmitoylation and cancer. Biochim. Biophys. Acta 1856, 107–120
26. Chamberlain, L. H., and Shipston, M. J. (2015) The physiology of protein S-acylation. Physiol. Rev. 95, 341–376
27. Fukata, Y., Murakami, T., Yokoi, N., and Fukata, M. (2016) Local palmitoylation cycles and specialized membrane domain organization. Curr. Top. Membr. 77, 97–141
28. Tian, L., Jeffries, O., McClafferty, H., Molyvadas, A., Rowe, I. C., Saleem, F., Chen, L., Greaves, J., Chamberlain, L. H., Knaas, H. G., Ruth, P., and Shipston, M. J. (2008) Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels. Proc. Natl. Acad. Sci. U.S.A. 105, 21006–21011
29. Stephens, G. J., Page, K. M., Bogdanov, Y., and Dolphin, A. C. (2000) The α1β1Ca²⁺ channel amino terminus contributes determinants for β subunit-mediated voltage-dependent inactivation properties. J. Physiol. 525, 377–390
30. Locke, D., Koren, V. I., and Harris, A. L. (2006) Isoelectric points and processing of ENaC subunits in the rat kidney. Annu. Rev. Physiol. 68, 77–141
31. Lock, D., Koren, V. I., and Harris, A. L. (2006) Isoelectric points and post-translational modifications of connexin26 and connexin32. Curr. Opin. Cell Biol. 18, 520–5210
32. Crane, J. M., and Verkman, A. S. (2009) Reversible, temperature-dependent supramolecular assembly of aquaporin-4 orthogonal arrays in live cell membranes. Biophys. J. 97, 3010–3018
33. Mueller, G. M., Yan, W., Copelovitch, L., Jarman, S., Wang, Z., Kinlough, C. L., Tolino, M. A., Hughay, R. P., Kleyman, T. R., and Rubenstein, R. C.
ENaC Activation by Specific Palmitoyltransferases

by inhibition of acyl-protein thioesterase enzymatic activities. PLoS One 8, e7523

53. Coleman, R. A., Rao, P., Fogelson, R. J., and Bardes, E. S. (1992) 2-Bromopalmityl-CoA and 2-bromopalmmitate: promiscuous inhibitors of membrane-bound enzymes. Biochim. Biophys. Acta 1125, 203–209

54. Heiler, S., Mu, W., Zöller, M., and Thu, N. (2015) The importance of claudin-7 palmitoylation on membrane subdomain localization and metastasis-promoting activities. Cell Commun. Signal. 13, 29

55. Van Itallie, C. M., Gambling, T. M., Carson, J. L., and Anderson, J. M. (2005) Palmitoylation of Claudins is required for efficient tight-junction localization. J. Cell Sci. 118, 1427–1436

56. Kiuchi-Saishin, Y., Gotoh, S., Furuse, M., Takasuga, A., Tano, Y., and Tsukita, S. (2002) Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J. Am. Soc. Nephrol. 13, 875–886

57. Treutlein, B., Brownfield, D. G., Wu, A. R., Neff, N. F., Mantalas, G. L., Espinoza, F. H., Desai, T. J., Krasnow, M. A., and Quake, S. R. (2014) Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. Nature 509, 371–375

58. Ohno, Y., Kihara, A., Sano, T., and Igarashi, Y. (2006) Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. Biochim. Biophys. Acta 1761, 474–483

59. Fukata, Y., and Fukata, M. (2010) Protein palmitoylation in neuronal development and synaptic plasticity. Nat. Rev. Neurosci. 11, 161–175

60. Rocks, O., Peyer, A., and Bastiaens, P. I. (2006) Spatio-temporal segregation of Ras signals: one ship, three anchors, many harbors. Curr. Opin. Cell Biol. 18, 351–357

61. Gaeggerer, H. P., Gonzalez-Rodriguez, E., Jaeger, N. F., Horisberger, J. D., and Rossier, B. C. (2005) Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. J. Am. Soc. Nephrol. 16, 878–891

62. Edinger, R. S., Bertrand, C. A., Rondandino, C., Apodaca, G. A., Johnson, J. P., and Butterworth, M. B. (2012) The epithelial sodium channel (ENaC) establishing a trafficking vesicle pool responsible for its regulation. PLoS One 7, e46593

63. Edinger, R. S., Coronnello, C., Bodnar, A. J., Labarca, M., Bhalla, V., LaFramboise, W. A., Benos, P. V., Ho, J., Johnson, J. P., and Butterworth, M. B. (2014) Aldosterone regulates microRNAs in the cortical collecting duct to alter sodium transport. J. Am. Soc. Nephrol. 25, 2445–2457

64. Heidrich, E., Carrattino, M. D., Hughly, R. P., Pilewski, J. M., Kleyman, T. R., and Myerburg, M. M. (2015) Intracellular Na+ regulates epithelial Na+ channel maturation. J. Biol. Chem. 290, 11569–11577

65. Chen, J., Myerburg, M. M., Passero, C. J., Winskari, K. L., and Sheng, S. (2011) External Cu+ inhibits human epithelial Na+ channels by binding at a subunit interface of extracellular domains. J. Biol. Chem. 286, 27436–27446

66. Chen, J., Ray, E. C., Yates, M. E., Buck, T. M., Brodsky, J. L., Kinlough, C. L., Winskari, K. L., Hughly, R. P., Kleyman, T. R., and Sheng, S. (2015) Functional roles of clusters of hydrophobic and polar residues in the epithelial Na+ channel knuckle domain. J. Biol. Chem. 290, 25140–25150

67. Al-bataineh, M. M., Gong, F., Marciszyn, A. L., Myerburg, M. M., and Pastor-Soler, N. M. (2014) Regulation of proximal tubule vacuolar H+-ATPase by PKA and AMP-activated protein kinase. Am. J. Physiol. Renal Physiol. 306, F981–F995

68. Gong, F., Alzamora, R., Smolak, C., Li, H., Naveed, S., Neumann, D., Hallows, K. R., and Pastor-Soler, N. M. (2010) Vacuolar H+-ATPase apical accumulation in kidney intercalated cells is regulated by PKA and AMP-activated protein kinase. Am. J. Physiol. Renal Physiol. 298, F1162–F1169