The epithelial sodium channel (ENaC) is ubiquitinated by the E3 ligase Nedd4-2 at the apical membranes of polarized cortical collecting duct (CCD) epithelial cells. This leads to ENaC endocytosis and possible degradation. Because ENaC is known to recycle at the apical membranes of CCD cells, deubiquitinating enzymes (DUBs) are likely involved in regulating ENaC surface density by facilitating ENaC recycling as opposed to degradation. Using a chemical probe approach to tag active DUBs, we identified ubiquitin C-terminal hydrolase (UCH) isoform L3 as the predominant DUB in endosomal compartments of CCD cells. Blocking UCH-L3 activity or reducing its expression by selective knockdown increased ENaC ubiquitination and resulted in its removal from the apical membranes of CCD cells. Functionally this caused a rapid reduction in transepithelial Na⁺ currents across the CCD epithelium. Surface biotinylation demonstrated the loss of ENaC from the apical surface when UCH-L3 was inhibited. Whole cell or apical surface immunoprecipitation demonstrated increased ENaC ubiquitination with UCH-L3 inhibition. This constitutes a novel function for UCH in epithelia and in the regulation of ion channels and demonstates the dynamic regulation of apically located ENaC by recycling, which is facilitated by this DUB.

The cell surface densities, endocytosis, and degradation of many transmembrane proteins are dynamically regulated by ubiquitination (1–4). The addition of ubiquitin to target proteins by E1 (activating), E2 (conjugating), and E3 (ligase) enzymes results in protein recognition, internalization, and processing by the endosomal sorting complexes required for transport, which may direct ubiquitin-tagged proteins to lysosomal degradation (5). Multi-ubiquitination elicits ENaC endocytosis and can lead to channel degradation (6–10). Recently, ENaC ubiquitination by the E3 ligase Nedd4-2 was demonstrated to occur at the plasma membrane (11). Deubiquitinating enzymes (DUBs) are proteases that remove single ubiquitin (Ub) moieties from an Ub chain or cleave the isopeptide bond between Ub and a lysine residue on the target protein to reverse the Ub addition and rescue proteins from a degradative fate (12–15). It has been recently reported that the DUB USP2–45 was up-regulated by aldosterone and implicated in deubiquitinating ENaC (16). To impact functional transepithelial sodium transport acutely, however, DUBs would be required to remove Ub from ENaC derived from the apical surface before the channel subunits were directed to degradation. This previous study provided no evidence for deubiquitination of ENaC derived from the apical surface, which was the aim of these investigations.

Our prior work has established the physical association of ubiquitinated ENaC with epsin, which facilitates clathrin-mediated endocytosis of the channel (17). In a separate study, we demonstrated the dynamic recycling of endocytosed ENaC back to the apical membrane of polarized CCD epithelial cells (18). In these investigations over 80% of the endocytosed ENaC was recycled to the apical membrane via a cAMP-regulated intracellular channel pool. These findings suggest that ENaC may be deubiquitinated in apical compartments to maintain a recycling channel population at the apical membranes.

Over 90 mammalian DUBs have been identified and classified into five families (12, 19). To identify the specific DUBs involved in ENaC regulation from this diverse group, we employed a chemical probe approach. Hemagglutinin (HA)-tagged Ub probes were engineered with a C-terminal modification incorporating a thiol-reactive group to act as a selective substrate for DUB interactions (20–22). Binding of DUBs to the active site cysteine of the probe facilitated stable covalent epitope tagging of these active DUBs. Several DUBs were isolated using this approach, with a predominant DUB in endocytic compartments of CCD cells identified as UCH-L3. Pharmacological inhibition of UCH, together with biochemical and siRNA techniques, confirmed the central role that UCH-L3 involved in ENaC regulation from this diverse group, we employed a chemical probe approach. Hemagglutinin (HA)-tagged Ub probes were engineered with a C-terminal modification incorporating a thiol-reactive group to act as a selective substrate for DUB interactions (20–22). Binding of DUBs to the active site cysteine of the probe facilitated stable covalent epitope tagging of these active DUBs. Several DUBs were isolated using this approach, with a predominant DUB in endocytic compartments of CCD cells identified as UCH-L3. Pharmacological inhibition of UCH, together with biochemical and siRNA techniques, confirmed the central role that UCH-L3 plays in acutely regulating ENaC surface density. UCH-L3 is required to maintain a stable apical membrane ENaC population by facilitating the dynamic recycling of ENaC at the apical surface.
UCH-L3 Regulates ENaC Recycling

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse CCD cells (mpkCCDc14) maintained between passages 20–35 were cultured on permeable filter supports (with 6.5-, 12-, or 75-mm diameters) in supplemented culture medium as previously described (18). Before use in electrophysiological experiments, the cultures were incubated in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium without additional supplementation for 24 h as described previously (18).

Short Circuit Current Measurements—mCCD cells cultured on filter supports were mounted in modified Costar Ussing chambers, and the cultures were continuously short circuited between passages 20–35 were cultured on permeable filter supports (with 6.5-, 12-, or 75-mm diameters) in supplemented culture medium as previously described (18). Before use in electrophysiological experiments, the cultures were incubated in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium without additional supplementation for 24 h as described previously (18).

Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium was used in Ussing chamber bathing Ringer’s 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. The chambers were constantly gassed with a mixture of 95% O2/5% CO2 at 37 °C, which maintained the pH at 7.4.

Clathrin-coated Vesicle (CCV) Preparations—CCVs were isolated by a modification of the method of Metzler et al. (24) and described previously by us for CCD cells (17). Briefly, CCD cells cultured on 75-mm filter supports were scraped and homogenized in isolation buffer containing 10 mM MES-NaOH, pH 6.5, 100 mM KCl, 1 mM EGTA, 0.5 mM MgCl2, 0.02% NaN3, with protease inhibitors, using a Dounce homogenizer and then sonicated by three bursts of 10 s on ice. The homogenate was centrifuged at 17,800 g for 1 h. The supernatant was aspirated, and 50 μl of 2× SDS sample buffer was added to the pellet, and samples were heated to 95°C for 2 min and loaded onto a gel for separation by SDS-PAGE.

PCR—To verify the expression of the UCH family of proteins in mCCD cells, reverse transcription-PCR was performed. Specific primer pairs for L1 (GCCATCGGCAAGATGCTAGC-AGC; GGACTAGACAAAACCCATCCC), L3 (CAGTCTGAGGACAACTGAGAC; TGCTATGCTGCAGGAGAGC), and L5 (CACCATGTCGACAAATGCGGG; GAAATTAGTGAGCACTGTGAC) were used to amplify the full-length cDNA from either mCCD cell library or mouse kidney cDNA library, which acted as a positive control. The product was separated on an agarose gel.

Surface Protein Biotinylation—Biotin labeling of membrane resident proteins were carried out on mCCD cells cultured on 12-mm-diameter filter inserts as described previously (18, 23). Biotin-labeled proteins were separated using streptavidin–conjugated beads, and samples were run on SDS-PAGE to determine the relative density of ENaC at the membrane surface of mCCD cells. Densitometric evaluation of the exposed blots was performed using Quantity One (Bio-Rad).

Ub Immunoprecipitation—To confirm the ubiquitinated state of ENaC, mCCD cells cultured on 75-mm filters were treated with UCH-L3 inhibitor for 3 h, with untreated filter cultured cells acting as controls. The cells were lysed in RIPA buffer, and equal amounts of protein were immunoprecipitated using an anti-Ub antibody as previously described (17). Isolated Ub-tagged proteins were separated by SDS-PAGE and Western blotted with anti-α, β, and γENaC antibodies (see below). The blots were densitometrically quantitated using Quantity One, and the fold increase in ubiquitinated α- and γ-ENaC is reported.

Biotinylation Followed by Ub Immunoprecipitation—To detect ENaC that was ubiquitinated at the cell surface, UCH-L3 inhibitor treated (3 h) and untreated 75-mm filters were surface biotinylated (as described above). Following separation using streptavidin beads, the samples were washed by centrifugation at low speed (10 000 × g) and resuspended in the biotinylation

Ubiquitin Probe Production, Cell Extract Labeling, and Detection—The procedure to produce HA-tagged UB probes has been described in detail previously (20–22, 25, 26). A vinyl methyl ester was employed as the electrophilic trap to covalently tag active DUBs because it had shown the broadest reactivity with a number of DUBs (20). Premanufactured probes were added either to post-nuclear supernatant (PNS) obtained by low speed (10 000 × g) centrifugation following cell lysis or CCV or EE preparations (as described above). For samples to be used directly in Western blotting 20 μg of cell lysate was incubated with 0.1 μg of manufactured probe for 1 h at 37°C. The samples were then separated by SDS-PAGE and blotted for using an anti HA antibody (Sigma). HA-tagged samples were immunoprecipitated using a monoclonal HA-conjugated-agarose antibody (Sigma). For IP the HA antibody-agarose suspension was washed by centrifugation (10 000 × g for 1 min) and resuspended in RIPA buffer used for cell lysis. Washed resin was added to the tagged samples and incubated at 4°C overnight on an orbital shaker. Following incubation, the samples were washed four times as above. On the final wash the supernatant was aspirated, and 50 μl of 2× SDS sample buffer was added to the resin, and samples were heated to 95°C for 2 min and loaded onto a gel for separation by SDS-PAGE.

Detection—The procedure to produce HA-tagged UB probes has been described in detail previously (20–22, 25, 26). A vinyl methyl ester was employed as the electrophilic trap to covalently tag active DUBs because it had shown the broadest reactivity with a number of DUBs (20). Premanufactured probes were added either to post-nuclear supernatant (PNS) obtained by low speed (10 000 × g) centrifugation following cell lysis or CCV or EE preparations (as described above). For samples to be used directly in Western blotting 20 μg of cell lysate was incubated with 0.1 μg of manufactured probe for 1 h at 37°C. The samples were then separated by SDS-PAGE and blotted for using an anti HA antibody (Sigma). HA-tagged samples were immunoprecipitated using a monoclonal HA-conjugated-agarose antibody (Sigma). For IP the HA antibody-agarose suspension was washed by centrifugation (10 000 × g for 1 min) and resuspended in RIPA buffer used for cell lysis. Washed resin was added to the tagged samples and incubated at 4°C overnight on an orbital shaker. Following incubation, the samples were washed four times as above. On the final wash the supernatant was aspirated, and 50 μl of 2× SDS sample buffer was added to the resin, and samples were heated to 95°C for 2 min and loaded onto a gel for separation by SDS-PAGE.

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lysate buffer three times. After the final centrifugation, the samples were resuspended in 25 µl of RIPA buffer with 10% β-mercaptoethanol and incubated for 30 min at 40 °C to disassociate the bound proteins from the streptavidin beads. The samples were centrifuged (13,000 × g) for 5 min to pellet the streptavidin beads, and the supernatant was collected and added to RIPA buffer to a final volume of 750 µl. This diluted the remaining β-mercaptoethanol over ~30-fold. Next samples were immunoprecipitated with the anti-Ub antibody as described above to detect biotinylated ENaC, which had been ubiquitinated. After IP, the samples were separated by SDS-PAGE and blotted for the three ENaC subunits.

**siRNA Knockdown**—A combination of three separate oligonucleotides (Santa Cruz Biotechnology) were used to specifically knock down mouse UCH-L3 expression in mCCD cells. The oligonucleotides were introduced by lipidic transfection as previously described according to manufacturer’s protocols (18). GAPDH siRNA was employed as a negative control to ensure that it did not effect UCH-L3 expression. Following transfection, the cells were seeded onto 6.5-mm filter inserts at superconfluency and allowed 48 h to form a tight transporting monolayer. The filters were mounted in modified Ussing chambers for cis–trans recording as described above.

**Chemicals, Reagents, and Antibodies**—UCH inhibitors, 4,5,6,7-tetrachloroindan-1,3-dione (UCH-L3 inhibitor) and ubiquitin C-terminal hydrolase L1 inhibitor (EMD Biosciences, San Diego, CA) were reconstituted in Me2SO at 1000-fold stock concentration just prior to addition in all investigations. All of the other reagents were obtained from Sigma unless stated. Anti-HA antibodies and resin-conjugated anti-HA-agarose antibody were obtained from Sigma. A mouse monoclonal βENaC and rabbit polyclonal γENaC antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal αENaC antibody was developed by us and previously reported and characterized for studies in the CCD cells (18). A rabbit polyclonal βENaC antibody kindly provided by Dr. M. Knepper (National Institutes of Health, Bethesda, MD) was used to test Ub labeling of βENaC, but data are not presented for this antibody. A rabbit polyclonal anti-UCH-L3 antibody was obtained from Santa Cruz Biotechnology. The rabbit polyclonal anti-ubiquitin antibody was obtained from Stressgen Bioreagents (Ann Arbor, MI).

**Statistics**—The summarized data are presented as the means and standard error (Sigmaplot 2000; SPSS). Differences determined by Student’s t test with p < 0.05 considered significantly different (Sigmaplot 2000).

**RESULTS**

**DUB Labeling Identifies UCH-L3 in Endosomes of mCCD Cells**—An HA-tagged Ub probe was employed to identify DUBs active in mCCD cells. Samples from PNS, CCV, and EE preparations isolated from mCCD cells were incubated with the chemical probe. Because only active DUBs will modify the Ub tag and become covalently HA-tagged, the procedure specifically identified active DUBs in these subcellular compartments. The proteins were separated by SDS-PAGE and blotted with anti-HA antibody to identify the HA-tagged DUBs (Fig. 1A). The specificity for the HA tagging was verified by the addition of the cysteine modifier N-ethylmaleimide during the labeling reaction, which eliminated DUB interactions with the HA probe. Several active DUBs were identified in this manner (note the different molecular mass bands) with a predominant labeled species (~40 kDa) evident in all of the samples. Tagged samples could be further isolated by immunoprecipitation

![FIGURE 1. Chemical labeling of UCH-L3 in mCCD cells.](image-url)

A, a vinyl methyl ester HA-Ub probe was used to covalently tag active DUBs in mCCD cells. The samples were incubated with the Ub probe following isolation of the CCV or EE compartments or PNS. Following DUB tagging, the samples were separated by SDS-PAGE and blotted using an anti-HA antibody. The addition of N-ethylmaleimide to disrupt the covalent binding of the HA tag to the active DUBs verified the specificity of the tagging reaction. B, immunoprecipitation using anti-HA-agarose antibody followed by immunoblotting for HA exposed the same DUB species separated in the initial screen (arrow; heavy (HC) and light chains (LC) are indicated). C, to verify that the isolated DUB was UCH-L3, the samples were immunoprecipitated as in Fig. 1B (VME-HA IP; lanes 1 and 2) and blotted with an anti-UCH-L3 antibody. An enrichment in UCH-L3 in both PNS and EE fractions was observed (arrow). As a control IP (Cont.; lane 3), a nonspecific antibody was used that failed to pull down UCH-L3. Western blotting the untagged PNS with the UCH-L3 antibody showed the native UCH-L3 in PNS (PNS WB; lane 4), which migrated faster on the gel as the UCH-L3 was not covalently attached to the HA-Ub tag. PNS samples that were immunoprecipitated (IP, as in lane 1) were blotted using an anti-HA antibody, which confirmed the molecular mass of the tagged UCH-L3 (lane 5). D, to identify the forms of UCH expressed in the mCCD cells, reverse transcription-PCR were carried out for UCH-L1, -L3, and -L5 using cDNA from mCCD (lanes C) and whole mouse kidney (lanes K, to act as a control). Only UCH-L3 was found to be expressed in mCCD cells.
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using an anti-HA antibody followed by separation by SDS-PAGE. This approach also identified the predominant lower molecular mass species (Fig. 1B). The position of this lower molecular mass DUB species was similar to that found in studies of malignant human cells using the same technique, which identified UCH family members at this position (27).

To confirm the identity of the DUB as an UCH isoform, HA-tagged samples from the PNS and EE preparations were immunoprecipitated using the anti-HA antibody and blotted for UCH-L3 (Fig. 1C, lanes 1 and 2). UCH-L3 detected in these samples was absent in immunoprecipitates from PNS samples that were immunoprecipitated using a nonspecific antibody (lane 3). Endogenous UCH-L3 was also detected by Western blot of untagged PNS material from mCCD cells (Fig. 1C, lane 4). Note that the band is of lower molecular mass when compared with the HA-tagged samples because the tagging procedure covalently added an HA-tagged ubiquitin moiety (~10 kDa) to the UCH-L3 DUB. Blotting the HA-tagged PNS with an anti-HA antibody after the IP produced a single band corresponding to the labeled UCH-L3, again attesting to the specificity of the reaction (Fig. 1C, lane 5).

The UCH-L3 isoform has been shown to be more widely expressed than UCH-L1, which is predominantly localized in neuronal tissues (14, 28–33). To determine which of the UCH isoforms are found in mCCD cells, reverse transcription-PCR was carried out using primers to detect UCH-L1, UCH-L3, and UCH-L5 (Fig. 1D). UCH-L3 expression in mCCD cells was confirmed, whereas both the UCH-L1 and UCH-L5 isoforms were not detected in mCCD cells but were present in the whole kidney samples, which acted as positive controls (29).

UCH-L3 Inhibition Reduces ENaC Currents—To determine whether UCH-L3 is implicated in the acute regulation of ENaC expressed endogenously in mCCD cells, a specific, cell-permeable UCH-L3 inhibitor (4,5,6,7-tetrachloroindan-1,3-dione) was employed to reduce the deubiquitinating activity of UCH-L3. This compound exhibits a greater potency for UCH-L3 inhibition than an isatin O-acyl oxime compound that acts as a reversible, competitive, active site-directed inhibitor of UCH-L3. This compound was used in previous studies by others (34). I_{SC} was measured across mCCD cells cultured on filter supports and mounted in modified Ussing chambers. The UCH-L3 inhibitor (10 μM) was added to cells under basal (unstimulated) conditions, before or during cAMP stimulation (10 μM forskolin). We have demonstrated previously that forskolin alters the distribution of ENaC from a subapical vesicle storage population and translocates channels into the apical membranes of these cells (18). Inhibitor addition at the peak of the forskolin stimulation resulted in a fairly rapid decline in ENaC I_{SC} (Fig. 2A) compared with vehicle-treated control cells, in which the I_{SC} remained stably elevated. The addition of amiloride at the end of the trace demonstrated that the majority of the recorded current was the result of ENaC-mediated Na^+ absorption. The results from five similar experiments, in which I_{SC} data were collected at 5-min intervals, are summarized in Fig. 2B. The values were normalized to those obtained prior to drug treatment. Under basal (unstimulated) conditions, inhibitor addition to both apical and basolateral bathing solutions caused the I_{SC} to decline but at a slower rate than in the presence of the cAMP agonist (Fig. 2C). The rate of current decline was significantly greater than that in vehicle-treated control cells, which displayed relatively stable values over the same period. The addition of the UCH-L3 inhibitor prior to forskolin stimulation resulted in a significantly reduced I_{SC} response and an accelerated rundown of ENaC current with a time course similar to that observed when UCH-L3 was inhibited at the peak of forskolin stimulation (data not shown).

To examine the specificity of the UCH-L3 versus UCH-L1 inhibitors, dose response relations for I_{SC} inhibition were determined for each compound using mCCD epithelia. The percentage current inhibition observed after 15 min of exposure for cells stimulated with forskolin was plotted over a range of concentrations (Fig. 2D). The specific UCH-L3 inhibitor abolished close to 80% of the current within 15 min at 100 μM compared with ~20% inhibition by the UCH-L1 inhibitor. The current inhibition for the UCH-L1 inhibitor at high concentrations was not significantly different from that at 10 μM, demonstrating that increasing concentrations did not have a deleterious impact on the mCCD cells and that UCH-L1 is not involved in maintaining ENaC activity.

UCH-L3 Inhibition Decreases Apical Membrane ENaC Expression—The requirement for a DUB in ENaC recycling at the apical surface highlights the dynamic nature of regulation achieved by the balance between channel ubiquitination and deubiquitination. To verify the loss of ENaC at the apical surface when UCH-L3 was inhibited, we biotin-labeled apical membrane proteins in polarized mCCD cells and determined the surface density of α, β, and γENaC by avidin affinity isolation and immunoblotting in UCH-L3 inhibited compared with control cells. In these experiments, the cells received no forskolin stimulation. The level of surface ENaC in UCH-L3 inhibited cells declined in a time-dependent manner compared with untreated controls, which remained stable (Fig. 3). There was no significant difference in the whole cell expression of the three ENaC subunits (whole cell lysate; Fig. 3A) over time in UCH-L3-treated versus control mCCD cells, suggesting that ENaC was lost from the apical surface rather than a generalized degradation of cellular ENaC subunits. Densitometric quantitation from two or more similar experiments (Fig. 3B) demonstrated that surface ENaC was essentially lost by 3 h when UCH-L3 was inhibited. Therefore, both electrophysiological and biochemical findings suggested that UCH-L3 was responsible for regulating the expression and recycling of endogenously expressed ENaC at the apical surface of mCCD cells.

UCH-L3 Inhibition Increases ENaC Ubiquitination—It has been previously demonstrated by us and others that ENaC is ubiquitinated by the action of Nedd4-2 (7, 10, 35). If UCH-L3 is specifically deubiquitinating ENaC retrieved from the apical membrane following Nedd4-2-dependent ubiquitination, inhibition of UCH-L3 should result in increased ubiquitination of ENaC subunits. To test this, mCCD cells cultured on large filter supports (75 mm in diameter) were treated with the UCH-L3 inhibitor for 3 h and compared with vehicle-treated controls. Equal protein concentrations of cell lysates were immunoprecipitated with an anti-Ub antibody, separated by SDS-PAGE, and blotted for α, β, and γENaC. A significant increase in ubiquitina-
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**Figure 2.** UCH-L3 inhibition reduces ENaC currents. A, representative short circuit current ($I_{SC}$) traces from mCCD epithelia. The cells were stimulated using 10 μM forskolin to increase intracellular cAMP levels that resulted in increased Na⁺ transport. The specific UCH-L3 inhibitor (10 μM) was added to both sides of the epithelial monolayer from a freshly prepared 1000X stock as indicated, causing the $I_{SC}$ to decline (gray trace). Control cells (black trace) received vehicle (Me2SO), which showed no significant effect on the $I_{SC}$. Amiloride addition (Amil, 10 μM) at the end of the trace confirmed that the majority of the recorded $I_{SC}$ was due to ENaC-mediated sodium transport. B, time course of current decline in forskolin-stimulated cells in the presence (closed triangles) or absence (open triangles) of UCH-L3 inhibition plotted as a percentage of maximal (forskolin-stimulated) current prior to inhibitor addition. C, time course of current decline in the presence (closed circles) and absence (open circles) of UCH-L3 inhibitor across unstimulated mCCD cells (basal $I_{SC}$). D, dose response relation for current inhibition by UCH-L3 (closed circles) and UCH-L1 (open squares) inhibitors. mCCD cells were stimulated with forskolin as in A, and the current inhibition after 15 min is plotted as a function of inhibitor concentration on a semi-log scale (n > 3 for each point).

**Figure 3.** UCH-L3 inhibition decreases apical ENaC labeling. A, mCCD cells were treated for increasing periods with the UCH-L3 inhibitor and surface biotinylation carried out to determine the surface density of α, β, and γENaC at the indicated time points. No significant change in the whole cell level of any ENaC subunit was detected, but a significant decrease in surface labeled ENaC was observed in cells where UCH-L3 was inhibited. B, densitometric quantitation of similar experiments as in A normalized to pretreated control levels (time 0) demonstrate a decline in ENaC surface density with UCH-L3 inhibition over time. Cont., control.

**Discussion.**

**Figure 4.** A, ubiquitinated ENaC was observed for αENaC and γENaC in UCH-L3 inhibitor-treated cells (Fig. 4A). In these experiments, however, we failed to detect a signal for βENaC (supplemental Fig. S1; see “Discussion”). Densitometric quantitation of at least two separate experiments demonstrated a 1.6 ± 0.2-fold increase in the level of ubiquitinated αENaC and a 2.3 ± 0.5-fold increase in ubiquitinated γENaC in cells that had been treated with the UCH-L3 inhibitor (Fig. 2B). The significant
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**DISCUSSION**

Several studies utilizing both overexpression systems and cells endogenously expressing ENaC have demonstrated that this channel is ubiquitinated by the action of the E3 ligase, Nedd4-2 (2, 9, 10, 17, 37). In addition, recent studies illustrated that ENaC was ubiquitinated by Nedd4-2 at the apical membrane (11, 37). The addition of ubiquitin moieties to the intracellular tails of the ENaC subunits induces rapid ENaC internalization and degradation. Although the relation of channel endocytosis to mono- versus poly- versus multi-ubiquitination remains unclear, engineered mono-ubiquitin constructs were sufficient to induce ENaC internalization in an exogenous expression system (11). This finding illustrated the significance of the Ub signaling pathway in the regulation of ENaC surface density.

We have previously defined the importance of ENaC recycling in the mCCD cell line, which endogenously expresses the channel, and this included a quantitative description of the rates of channel biosynthesis, recycling, and degradation (18). A recent study using epitope-tagged ENaC subunits expressed exogenously in polarized MDCK1 cells has further underscored the dependence of apical channel density on the ability of cells to insert channels from a subapical pool (38). The return of Ub-modified ENaC to the apical surface following its Nedd4-2-dependent retrieval requires the removal of ubiquitin moieties so that ENaC can enter recycling pathways and avoid trafficking (e.g. the endosomal sorting complexes required for transport).
pathways that would lead to its degradation (5). One or more of the large cohort of DUBs already described in mammalian cells was likely to perform this function, and we therefore sought to identify the DUB(s) involved in ENaC recycling. The tagged chemical probe approach that we employed specifically labeled active DUBs in fractions derived from mCCD cells and enabled the labeling of several ubiquitin-interacting proteins to be identified in clathrin-coated and early endosome membranes, compartments known to be involved in apical ENaC retrieval (17). A predominant protein was consistently labeled in endocytic fractions and was identified by PCR and Western blotting as UCH-L3. Although there is no previously reported role for UCH in epithelial cells, the UCH-L3 isoform is widely expressed, and in one of the first studies to characterize its the expression, UCH-L3 was clearly present in tissues where regulated ENaC trafficking is known to contribute to the control of channel activity (e.g. kidney, lung, gut) (28).

It is clear from the HA tagging procedure that more than one active DUB was isolated (Fig. 1, A and B). It is certainly likely that more than one DUB will be involved in deubiquitinating ENaC at different subcellular locations (e.g. ER versus endosomes), as is evident from the previous study by Fakitsas et al. (16), which demonstrated a role for USP2–45 in ENaC regulation using the same CCD cell line. It is also a possibility that more than one DUB may be required to deubiquitinate recycled ENaC at several points in the recycling pathway from endocytosis back to the apical membrane surface; however, we have not investigated the significance of the other identified active DUBs on ENaC regulation in this study.

Because of potential chemotherapeutic applications, several compounds have been developed that specifically target members of the UCH family, at submicromolar concentrations (34). We chose to employ two well characterized inhibitors specific for UCH-L3 and UCH-L1 to investigate the role of these isoforms in ENaC recycling. By PCR analysis it is clear that the UCH-L1 isoform is not expressed in the mCCD cells, consistent with its reported predominant expression in neuronally derived tissues (29). Moreover, incubation of increasing doses of the UCH-L1 inhibitor showed only a minor inhibition of ENaC-mediated sodium absorption that was not dose-dependent, whereas inhibition of the amiloride-sensitive current was readily observed when the specific UCH-L3 inhibitor was employed. This agrees with a reported ~28-fold greater selectivity of this compound for UCH-L1 over UCH-L3. Conversely, the UCH-L3 inhibitor has a reported IC50 = 0.6 μM and a ~125-fold greater selectivity for UCH-L3 over UCH-L1. Incubation of the mCCD cells with the UCH-L3 inhibitor resulted in a steady decline in I SC, reflecting the restriction of Ub-tagged channels from the recycling vesicle population. The kinetics of inhibition by the UCH-L3 inhibitor was more rapid in cells

TRACES FROM MCCD CELLS SUBJECTED TO GAPDH (BLACK TRACE) OR UCH-L3 KNOCKDOWN. The cells were stimulated with 10 μM forskolin and inhibited by amiloride (Amil, 10 μM) at the end of the trace to determine the contribution of ENaC to the total recorded I SC. A marked reduction in both basal and stimulated I SC was observed in the UCH-L3 knockdown cells. C, the ENaC-mediated (amiloride-sensitive) I SC from five separate experiments demonstrates that ENaC-mediated sodium transport in the UCH-L3 knockdown cells is significantly inhibited (~80%; p = 0.004) when compared with controls.
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where ENaC trafficking to the plasma membrane was stimulated by forskolin. The findings indicate that UCH-L3 is required for maintaining the cell surface density of ENaC during both basal and forskolin-stimulated conditions. cAMP stimulation elicits a more rapid recycling of ENaC, making the role of UCH-L3 during forskolin stimulation critical in maintaining steady-state ENaC density at the apical membrane. Recent preliminary findings using FM dyes to label the apical membrane under basal and stimulated conditions are consistent with this concept.3

Similar findings have emerged from studies of insulin-regulated GLUT4 traffic, which suggest that the transporter turns over constitutively, that differential changes in trafficking rates lead to mobilization of GLUT4 from intracellular compartments to the plasma membrane, and that the rate of turnover can be modulated by insulin signaling (39). The increased rate of current decline in the presence of forskolin is consistent with a cAMP-dependent increase in the kinetics of channel transit through the recycling pathway. Therefore, blocking ENaC deubiquitination elicits a more rapid decline in current.

In an attempt to demonstrate the ubiquitination of ENaC biochemically, we performed IPs using an anti-Ub antibody on cells treated with the UCH-L3 inhibitor for 3 h and compared these to samples without UCH-L3 treatment. A significant increase in the levels of ubiquitinated ENaC was observed in UCH-L3 inhibited cells. We were unable to detect any signal for βENaC, however, having employed both monoclonal and polyclonal antibodies in the Western blots. This negative result could be due to a lack of βENaC ubiquitination, as previously reported (9), or to inadequate sensitivity of the antibodies to detect a low level of ubiquitinated βENaC. Nevertheless, both α and γENaC exhibited greater levels of ubiquitination when UCH-L3 was inhibited.

Likewise we were able to detect an increase in the level of ubiquitinated ENaC derived from the cell surface. In these studies, membrane proteins were biotinylated, as before, isolated by incubation with streptavidin beads, and then subjected to the IP protocol using the same anti-Ub antibody. These results are consistent with a previous report of Nedd4-2-mediated ubiquitination of ENaC at the apical surface (11, 37) and suggest that inhibition of UCH-L3 preserves the ubiquitination of apically derived ENaC. Because UCH-L3 was detected in both CCV and EE preparations, it is likely that deubiquitination of ENaC would occur in early endosomal compartments, following internalization, and before the channels are diverted to degradation pathways. Although it was not directly tested, it is possible that UCH-L3 acted on ubiquitinated ENaC on the mem-

brane surface; however, immunofluorescent labeling studies showed no enrichment of UCH-L3 signal at the membrane and would argue against this notion (data not shown).

A more direct test of the involvement of UCH-L3 in ENaC regulation utilized siRNA constructs to specifically knock down this DUB. The results demonstrated a significant ~80% decrease in endogenous ENaC-mediated Na+ currents across mCCD epithelia in cells where UCH-L3 expression was reduced. These findings support the pharmacological inhibition observations and attest to the specificity of the compounds employed. Without the ability to rescue ubiquitinated channels from degradative pathways, the majority of the surface ENaC was lost, and transepithelial sodium transport was nearly abolished.

To our knowledge this is the first report of the involvement of a UCH in ion channel regulation, and it defines a novel role for this DUB in epithelial cells. UCH-L3 appears to be critically involved in ENaC recycling and is therefore a major component in determining steady-state levels of membrane surface ENaC, particularly during cAMP-mediated stimulation of membrane turnover. It remains to be determined whether UCH-L3 impacts the turnover of other ion channels known to be regulated by ubiquitination and recycling.

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