The Epithelial Sodium Channel (ENaC) Establishes a Trafficking Vesicle Pool Responsible for Its Regulation

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Abstract

The epithelial sodium channel (ENaC) is the rate-limiting step for sodium reabsorption across tight epithelia. Cyclic-AMP (cAMP) stimulation promotes ENaC trafficking to the apical surface to increase channel number and transcellular Na⁺ transport. Removal of corticosteroid supplementation in a cultured cortical collecting duct cell line reduced ENaC expression. Concurrently, the number of vesicles trafficked in response to cAMP stimulation, as measured by a change in membrane capacitance, also decreased. Stimulation with aldosterone restored both the basal and cAMP-stimulated ENaC activity and increased the number of exocytosed vesicles. Knocking down ENaC directly decreased both the cAMP-stimulated short-circuit current and capacitance response in the presence of aldosterone. However, constitutive apical recycling of the Immunoglobulin A receptor was unaffected by alterations in ENaC expression or trafficking. Fischer Rat Thyroid cells, transfected with αβγ-mENaC had a significantly greater membrane capacitance response to cAMP stimulation compared to non-ENaC controls. Finally, immunofluorescent labeling and quantitation revealed a smaller number of vesicles in cells where ENaC expression was reduced. These findings indicate that ENaC is not a passive passenger in regulated epithelial vesicle trafficking, but plays a role in establishing and maintaining the pool of vesicles that respond to cAMP stimulation.

Introduction

There is a tightly organized regulation of membrane proteins in polarized cells that helps to establish and maintain polarity and facilitate vectorial responses to internal and external cues. The extensive studies involving both neurons and epithelia demonstrate a degree of similarity in their ability to differentially organize proteins to specific membrane locations [1,2]. In epithelial cells distinct apical and basolateral membrane domains are maintained by junctional proteins that separate transport and regulatory proteins and organize proteins to these different membrane locations [3]. Like a number of other epithelial ion channels, the epithelial sodium channel (ENaC) is trafficked and faithfully delivered to the apical membrane of epithelial cells in which it is expressed [4–7].

The intracellular mechanisms involved in ENaC’s regulation by trafficking have been recently reviewed [3,8,9]. ENaC is delivered to the apical membrane via the biosynthetic pathway in two forms, both proteolytically cleaved (fully mature/active) and uncleaved (unprocessed) [10–15]. Once ENaC is delivered and inserted into the apical membrane a defined path has been described for the channel’s internalization and recycling [16–21]. In previous work we extensively characterized the trafficking of ENaC in a model mouse cortical collecting duct (mpkCCD) cell line to demonstrate the role of an intracellular storage pool that was mobilized by cAMP stimulation to increase ENaC density in the apical surface of the cells [22].

ENaC is retrieved from the apical membrane via clathrin mediated endocytosis in a process dependent on ubiquitylation of the channel [23–26]. ENaC then traffics to EEA1 (early endosome antigen 1)-positive early endosomes [25]. At this early stage a fate decision is made between degradation and recycling. Some ubiquitylated channels interact with Hrs and ESCRT pathway proteins and are targeted for lysosomal degradation [16] but the majority of ENaC is recycled in the mpkCCD cells, through a Rab11b-positive compartment, to maintain steady-state apical membrane channel number [27,28]. The role of deubiquitylating enzymes (DUBs) in this recycling has been demonstrated, and we previously investigated the impact of cAMP stimulation on ENaC turnover when DUBs were inhibited [17,29]. Results from these studies suggested that while ENaC is likely constitutively recycled at the apical membrane, there was a more rapid exocytic delivery and matched endocytic retrieval in the presence of cAMP stimulation.

Here we report that by removing hormonal and steroid supplementation from the cell culture media that the ENaC expression was significantly reduced. In conjunction with the reduction in ENaC expression the trafficking response to cAMP stimulation was also smaller. This CAMP response returned when...
ENaC expression was restored with replacement of the mineralocorticoid, aldosterone. It was unclear whether the change in vesicle compartment size was due to ENaC expression or some other protein/s that had been induced by aldosterone, so we specifically knocked down ENaC expression while maintaining aldosterone stimulation. Under these conditions the compartment size was again reduced. Inhibiting the activity of ENaC by preventing proteolytic cleavage did not alter the size or responsiveness of the trafficking vesicle pool. Introduction of ENaC into non-native ENaC-expressing epithelia recapitulated this trafficking compartment. These findings in conjunction with the membrane labeling and trafficking assays indicate that ENaC is capable of establishing and maintaining an intracellular vesicle population that is responsive to cAMP stimulation and required to acutely traffic ENaC to the apical surface. This study further differentiates the complex trafficking and recycling regulation observed in polarized epithelial cells from non-polarized and more general cell trafficking models, and underscores the value of performing these studies in polarized models.

Materials and Methods

Reagents and Antibodies

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated. Antibodies used included anti-actin (Sigma), anti-SGK (serum and glucocorticoid-induced kinase) (Cell Signaling Technology, Beverly, MA) and anti-ENaC antibodies (StressMarq, Victoria, BC, Canada). Membrane labeling was performed using the fixable lipophilic dye, FM1-43FX (Invitrogen). A cell permissive pro-protein convertase inhibitor, furin convertase inhibitor (FCI) (Alexis Biochemical/Enzo, Farmingdale, NY) was reconstituted in sterile water at 100 mM and placed in the culture medium of cells at a final concentration of 100 μM to inhibit the action of intracellular proteases (furin) that proteolytically cleave and activate ENaC.

Cell Culture

The mpkCCDc14 cells (provided by A. Vandewalle and M. Bens, Institut National de la Santé et de la Recherche Médicale, Paris, France) were grown in flasks (passage 30–40) in defined (supplemented) medium as described previously [22,28]. Growth medium was composed of equal volumes DMEM and Ham’s F12 supplemented with 60 mM sodium selenate, 5 mg/ml transferrin, 2 mM glutamine, 50 mM dexamethasone, 1 mM triiodothyronine, 10 ng/ml epidermal growth factor, 5 g/ml insulin, 20 mM D-glucose, 2% vol/vol FCS, and 20 mM HEPES (Invitrogen, Sigma), pH 7.4, at 37°C in 5% CO2. The medium was changed every second day. For all experiments, the mpkCCD cells were subcultured onto permeable filter supports (0.4 μm pore size, 0.33 cm2 or 4 cm2 surface area; Transwell, Corning, Lowell, MA). The recycling of radiolabelled IgA has been performed previously by us on polarized epithelial cells and described in several studies [30–36]. In brief, 125I-IgA was iodinated using the ICI method to a specific activity of 1.0–2.0×107 cpm/μg. Filter-grown mpkCCD cells expressing wild-type plgR were maintained in unsupplemented or fully supplemented media (described above), and 125I-IgA was internalized from the apical cell surface of the cells for 10 min at 37°C. Following ligand internalization the apical surface of the cells were treated three times for 10 min with 25 μg/ml trypsin at 4°C to remove cell surface bound ligand. The cells were then treated with 125 μg/ml soybean trypsin inhibitor for 10 min at 4°C. The cells were rapidly washed three times, the apical and basolateral medium aspirated, and replaced with fresh medium. The cells were then incubated for 3 min at 37°C. This wash procedure takes 5 min at 37°C. Fresh medium was added to the cells, and they were chased for up to 2 h at 37°C. At designated time points, the apical and basolateral media (0.5 ml) were collected and replaced with fresh media. After the final time point, filters were cut out of the insert and the amount of 125I-IgA quantified with a γ counter. The media samples were precipitated with 10% trichloroacetic acid for 30 min on ice, and then centrifuged in a microcentrifuge for 15 min at 4°C. The amount of 125I-IgA in the trichloroacetic acid-soluble (degraded) and insoluble fractions (intact) was quantified with a γ counter. An
equal number of cells (not expressing the plgR) were treated identically, and these values were subtracted from those cells expressing the plgR. The extent of apical recycling was expressed as a percentage of apical signal at time 0 for cells cultured in supplemented versus unsupplemented medium.

Apical membrane labeling and vesicle endocytosis

To specifically label apical membrane mkpCCD cells cultured on filter supports were first washed in PBS containing 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ (PBS+CM) and then in a Ringer’s solution (as above). The apical solution was replaced with Ringers solution containing FM1-43-FX at a concentration of 50 µg/ml which remained on the cells for 0, 2, 5 or 10 minutes. Cells were either maintained under basal (unstimulated) conditions or were stimulated with forskolin (10 µM). At the chosen time points, cells were rapidly washed four times in cold (4°C) Ringer’s solution to remove excess dye and wash off remaining apical membrane label (that had not been internalized) and immediately fixed in a cold (4°C) paraformaldehyde buffer (4% in PBS at pH7.4). Following 30 min fixation at 4°C, cells were washed in cold PBS+CM three times, and nuclei were counterstained using Hoechst 33342 (trihydrochloride, 10 nM) cell permeant nuclear dye (Invitrogen) for 10 min. Cells were washed thrice in PBS+CM and mounted onto slides using Fluoromount-G (SouthernBiotech, Birmingham, AL) for imaging, as previously described [28]. Images were captured using an Olympus IX81 fluorescent microscope (Olympus, Center Valley, PA) fitted with a DSU spinning disk and 300 W fluorescent light source using a 60X, 1.4 N.A. oil objective. Single fluorescent images were captured using a Retiga cooled CCD camera (QImaging, Surrey, BC, Canada) at 1024X1024 resolution using SlideBook (Olympus). Linear adjustments of brightness and contrast were made offline in MetaMorph (Molecular Devices Corp, Downingtown, PA). 3-D reconstructions of spinning disk optical sections were carried out using MetaMorph. Vesicle counts from the image stacks were obtained after image threshold and object counting in MetaMorph, and an average vesicle count through the whole image stack was calculated for >50 cells for at least 2 fields on each slide. The vesicle counts for 3 separate experiments were averaged to produce a vesicle number per cell (based on nuclei labeling).

siRNA

To knock down the expression of βENaC, siRNA was commercially obtained (Dharmacon/Thermo Fisher Scientific, On-Target Plus) and a pool of 4-siRNAs were introduced into the mkpCCD cells using lipofectamine 2000 at a concentration of 50 nM as described previously [17,28][3]. The target sequences for βENaC were GCCUGGAUGUUUGCAUAAA, GCCAUGUG-GUUCCUGCUUA, CACUGGAACUUACGCCUA and GACCGAGGCUAGCAUCA. Cells were seeded onto filter supports and allowed to polarize over 72 hrs before use in electrophysiological experiments. Following ISC and CT measurements the cells were harvested from the filter supports in lysis buffer (62.5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, 0.7% Triton-X100, 1.5%NP40, pH 8, containing protease inhibitors) and proteins were resolved on 6–18% SDS-PAGE gels, transferred to PVDF [1 hr at 100 V], and blotted for proteins of interest to determine the extent of protein knockdown. Desiometric quantification of protein band intensities was carried out in Adobe Photoshop CS5.1 (Adobe, San Jose, CA) and values were expressed as a percentage of control signal, following background subtraction, and normalization to total protein expression (actin).

Statistics

All data were analyzed using SigmaPlot (Systat, Chicago, IL). Summarized data were evaluated for normality and equal variance, and t-tests were carried out to determine whether differences were statistically different from each other. For any difference in the mean values, $P<0.05$ was considered significantly different.

Results

ENaC Expression and I$_{Na}$ decreases without mineralocorticoid supplementation

Figure 1A illustrates typical short-circuit current (I$_{SC}$) and transepithelial capacitance (C$_T$) traces from mkpCCD epithelia cultured in fully supplemented media and stimulated with 10 µM forskolin. The complete media used to culture the mkpCCD cells is supplemented with a mixture of hormones and trace metals which includes 50 nM dexamethasone (dex) to maximize ENaC expression (see methods). The addition of forskolin (10 µM) to the basolateral bathing chamber induces an increase in measured I$_{SC}$ and C$_T$. We previously characterized this response to show the I$_{SC}$ increase was due to the insertion of ENaC-containing vesicles into the apical membrane, which increased both channel number (as determined by surface biotinylation) and apical membrane surface area as measured by membrane capacitance (C$_T$) [22,37]. When the cells are cultured in an unsupplemented medium (as described in the methods) without hormones or steroids, ENaC expression is reduced (Figure 1B) and the amiloride-sensitive I$_{SC}$ is significantly smaller under both basal and cAMP-stimulated conditions (Figure 1B). In conjunction with this loss in ENaC-mediated current there is a reduction in the membrane capacitance response with forskolin stimulation (Figure 1B, and summarized in Figure 1C). The reduction in ΔC$_T$ stimulation in the absence of dex supplementation suggests a smaller number of vesicles were exocytosed in response to cAMP stimulation along with the reduction in ENaC expression.

Aldosterone restores ENaC expression and cAMP response

To examine the possibility that the cells cultured in basic media were primarily affected by the removal of steroids from the media, they were treated with increasing concentrations and over increasing time with aldosterone (Figure 2A). A significant increase in ENaC-mediated I$_{SC}$ was observed after 6 hours of aldosterone treatment and by 12 hours both basal and cAMP stimulated I$_{SC}$ had returned to control levels (Figure 2A,C). The maximal increase in I$_{SC}$ was typically observed by 24 hours with no significant increase in ENaC currents after 24 hours or above concentrations of 100 nM. We previously reported on the time course of aldosterone (aldo) stimulation in the mkpCCD cells and these I$_{SC}$ results are in agreement with the previously published results [38,39]. All subsequent stimulation experiments therefore employed a 100 nM, 24 hour aldosterone stimulation.

In conjunction with the increase in amiloride-sensitive I$_{SC}$ with aldosterone stimulation, the change in C$_T$ with forskolin stimulation also increased. After a 24 hour aldosterone stimulation the ΔC$_T$ and ΔI$_{SC}$ response to cAMP stimulation were significantly greater than cells that remained in unsupplemented medium (Figure 2B). Therefore as ENaC expression increased there was a larger intracellular vesicle pool of ENaC available for insertion into the apical membrane after cAMP stimulation (Figure 2C). By combining the data for all recordings, in both supplemented and unsupplemented media, the summarized graph (Figure 2D) indicates a smaller ΔI$_{SC}$
response to forskolin is associated with a reduced capacitance response.

ENaC knockdown reduces the cAMP-induced capacitance increase

As the expression of ENaC was altered by changing the steroid hormone supplementation of the cells it was possible that the change in C_T response was due to the alteration in expression of an aldosterone-induced protein involved in vesicle trafficking and not ENaC itself. To directly test if ENaC mediates the C_T response we used an RNAi knockdown approach to reduce the expression of βENaC in the mpkCCD cells cultured in the presence of aldosterone. The knockdown of βENaC resulted in a reduction of all three ENaC subunits (Figure 3A), and a decline in both basal and cAMP-stimulated amiloride-sensitive ISC (sample traces Figure 3B). The loss of ENaC-mediated Na⁺ transport was paralleled by a reduction in the basal and cAMP-stimulated C_T (Figure 3B). A summary of a number of similar experiments is presented in Figure 3C which plots the ISC and C_T responses to cAMP stimulation.

Reducing ENaC activity does not disrupt vesicle trafficking

While the knockdown results suggested that the number of ENaCs expressed were responsible for establishing the size of the vesicle pool trafficked in response to cAMP signaling, we next sought to determine if the activity or cleavage state of ENaC altered the capacitance response to cAMP stimulation. A number of proteases have been reported to cleave both α- and γ-ENaC and are required to achieve a fully active channel and maximal sodium transport [10,11,24,40–53]. Recent reports have suggested that cleaved ENaC may be regulated differently from the uncleaved channels [54]. We blocked full proteolytic cleavage of ENaC by using a pro-protein convertase inhibitor FCI. In cells where the activity of ENaC had been reduced by preventing full proteolytic activation, both basal and stimulated ISC was reduced (Figure 4A). The reduction in ISC was similar to that observed in βENaC knockdown or cells cultured in unsupplemented medium (compare Figure 4C with Figure 3C). However, unlike with βENaC knockdown, the change in C_T with cAMP stimulation was not significantly altered by the reduction in ENaC activity. To confirm the presence of uncleaved ENaC at the apical surface of mpkCCD cells, trypsin (1 mM) was added to the apical Ussing hemi-chamber to acutely activate uncleaved ENaC following cAMP stimulation, (see sample trace Figure 4B). A rapid increase in ISC was observed following trypsin addition indicating that a pool of uncleaved ENaC was present in the apical membrane. The summary plot of changes in INa against the change in C_T is presented in Figure 4C.

These data indicate that it is the expression rather than the activity or cleavage state of ENaC that determines the size of the vesicle pool which responds to cAMP stimulation. Sodium transport through ENaC does not impact the CT recording. As confirmation of this, the addition of amiloride at the end of each trace to block ENaC does not significantly alter the CT recordings (see all sample traces), indicating that the CT changes recorded are the result of changes to the membrane surface area which is not influenced by ENaC activity.

ENaC expression in FRT cells induces a trafficking vesicle population

As knockdown of ENaC reduced the C_T response to cAMP stimulation suggesting that ENaC expression was regulating the size of this vesicle pool, it was reasonable to hypothesize that introduction of ENaC into epithelial cells which do not highly express the channel may induce the formation of a trafficking vesicle pool. To test this we employed the Fisher Rat Thyroid (FRT) epithelial cell line which has been used previously by a number of researchers to investigate the function and trafficking of ENaC [14,54–57]. These cells do not natively express detectable levels of α,β,γ-ENaC, are readily transfected to express functional

Figure 1. ISC and C_T recordings with and without hormonal supplementation. (A) Representative ISC (black trace) and C_T (grey trace) recording from mpkCCD cells mounted in modified Ussing chambers and stimulated with (10 μM) forskolin. Addition of 10 μM amiloride at the end of the trace demonstrated the majority of the recorded ISC was Na⁺ transport via ENaC. (B) A similar trace from mpkCCD cells cultured in the absence of dexamethasone supplementation. (C) Summarized data for stimulated amiloride-sensitive current (INa) and C_T response to forskolin stimulation (n = 14) in cells with (+) and without (-) full supplementation.

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ENaC. The FRT cells can be cultured on filter supports where they form a polarized transporting epithelial monolayer. To investigate the capacitance changes occurring at the apical surface of control and ENaC transfected FRT cells, filters were mounted in the Ussing chambers in an apical to basolateral Na\(^+\) gradient and the basolateral membranes permeabilized using 50\(\mu\)M nystatin. We have employed this technique previously to isolate membrane domains and record changes in capacitance at the apical membrane [22]. Under these conditions a readily detectible amiloride-sensitive ISC was observed in ENaC transfected cells which could be stimulated by forskolin and fully inhibited by the specific ENaC blocker amiloride (Figure 5A). As the basolateral membranes were permeabilized, capacitance measurements reflected only changes occurring in the apical membrane (CA). ENaC-dependent current was absent in the control cells (see traces Figure 5A and summary Figure 5C). A small apical capacitance increase was recorded in control cells following cAMP stimulation (Figure 5C,D). The CA response was however significantly greater in FRT cells transfected with α,β,γ-ENaC compared to non-ENaC expressing controls indicating a larger pool of vesicles was trafficked in response to cAMP stimulation.

Constitutive recycling is not impacted by altering ENaC expression

We have previously demonstrated that the increase in ENaC activity and C\(r\) response to cAMP stimulation in mpkCCD cells is representative of a regulated recycling of the channel [22]. Withdrawal of forskolin results in a reversal of I\(sc\) and C\(r\) responses and cells can be repeatedly challenged with cAMP to elicit rounds of insertion and retrieval of ENaC [22]. Studies with cycloheximide inhibition of new channel synthesis and specific apical blockers have demonstrated that repetitive stimulations involve recycling of the apical channels rather than supplementation from the biosynthetic pool [22]. Here, we noted that while ENaC expression altered the size of the vesicle pool exocytosed in response to cAMP stimulation, the capacitance response is not entirely eliminated when ENaC expression was reduced by a removal of hormonal supplementation or specific siRNA knock-down. The cAMP stimulation still induced an increase in C\(r\) in unsupplemented mpkCCD or ENaC knockdown cells. There was also a small C\(r\) response in the untransfected FRT cells. It is therefore likely that an apical recycling vesicle compartment remains intact in the absence of ENaC expression. To test this we investigated the recycling of IgA in the mpkCCD cells.

Transferrin receptor or IgA recycling is often employed to investigate the mechanics of cargo turnover in model cell lines. As transferrin receptors are not expressed on the apical surface of mpkCCD cells, we chose to investigate the constitutive recycling of IgA by using the polymeric immunoglobulin receptor (pIgR). pIgR was transfected into mpkCCD cells and radiolabeled IgA used to assay the apical recycling of pIgR in cells with and without aldosterone supplementation. From the supplemental figure (figure S1) it is clear that the majority of the IgA was recycled back to the apical surface. There was no difference in the rate or extent of IgA

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**Figure 2. Aldosterone restores I\(sc\) and C\(r\) responses to cAMP stimulation.** (A) Cells in unsupplemented media were incubated with 100 nM aldosterone for increasing time and stimulated with forskolin. The basal (–cAMP) and forskolin stimulated (+cAMP) currents from cells with no aldosterone (unstimulated), 6, 12 and 24 hr aldosterone stimulation were compared to cells which received fully supplemented medium (supplemented). The average I\(sc\) response to cAMP stimulation is plotted as a bar graph on the right (n = 16). (B) The change in capacitance (ΔC\(_{r}\)) versus change in amiloride-sensitive I\(sc\) (ΔI\(sc\)) in response to forskolin stimulation is summarized for cells without supplementation and 24 hr stimulation with aldosterone. Both I\(sc\) and C\(r\) responses were significantly greater in the aldosterone treated cells compared to unsupplemented controls (n = 16, p < 0.01). (C) The change in capacitance versus total stimulated ENaC I\(sc\). The ΔI\(_{sc}\) and ΔC\(_{r}\) of the fully supplemented cells was not significantly different from cells treated with aldosterone for either 12 hr or 24 hrs, but these 3 groups were significantly greater than cells that received no supplementation (p < 0.01). (D) Data for all recordings were pooled (regardless of supplemented state) and categorized into groups that responded to cAMP with 0–5, 5–10 and >10 μA/cm\(^2\)ΔI\(_{sc}\). The average ΔC\(_{r}\) was then plotted for each group. A significantly larger capacitance response was observed in cells with ΔI\(_{sc}\) greater than 10 μA/cm\(^2\) compared to those with less than 5 μA/cm\(^2\). doi:10.1371/journal.pone.0046593.g002
recycling in cells without supplementation compared to the fully supplemented cells. These data indicate that constitutive apical recycling remains intact in cells receiving no supplementation, where ENaC expression has been reduced, and that the constitutive apical recycling compartments are unaffected by the alteration in ENaC expression. The cAMP-responsive pool of ENaC appears to reside in a vesicle compartment that is regulated separately from the constitutive apical recycling compartments responsible for IgA recycling.

Membrane labeling demonstrates a larger vesicle population with ENaC expression

The C\textsubscript{T} measurements from cells cultured without supplementation indicated a significant decrease in the number of vesicles that traffic ENaC to the apical membrane in response to cAMP stimulation. In order to maintain a steady state membrane surface area, the rate of endocytosis needs to match the exocytic rate. The capacitance recordings indicate that under basal conditions the membrane surface area remains stable. Following the addition of forskolin and cAMP stimulation there is an increase in C\textsubscript{T} which reaches a plateau by 10 minutes of forskolin stimulation (see sample traces Figure 1A and Figure 5). At this point the C\textsubscript{T} is again at steady state and thus the numbers of vesicles being internalized must match those being exocytosed. Under steady state conditions it should therefore be possible to use the endocytic rate or number of endocytosed vesicles as a measure of the corresponding vesicle exocytosis. We made use of the fixable membrane labeling fluorophore, FM1-43-FX to label endocytic vesicles. FM1-43-FX was included in the apical bathing medium over increasing time periods under basal conditions (unstimulated), and in the presence of forskolin. Representative confocal images from cells cultured with and without supplementation under these experimental conditions are presented in Figure 6. It is apparent from the images (Figure 6A&B) that there is a reduction in vesicle number in cells cultured in basic media compared to aldosterone stimulated cells. To quantitate the vesicle numbers at each time point, optical sectioning was performed using a spinning disk confocal microscope to produce 3-D images of the cell monolayer. The total vesicle counts were obtained from at least 50 cells in 2 fields of view from 3 separate experiments (average number of cells per time point = 247.7 ± 14.6) and normalized to control cells without forskolin stimulation at time 0 min (Figure 6C). Two time points are presented, 0 and 10 minutes. There was a significantly smaller number of internalized vesicles in cells cultured without

![Figure 3. ENaC siRNA reduces I\textsubscript{SC} and cAMP C\textsubscript{T} response.](image)

(A) Western blots of whole cell lysate from βENaC knockdown demonstrate reduction in expression of ENaC compared to control siRNA transected mpkCCD cells. The actin-corrected percent reduction in expression (n = 3) are summarized to the right of each representative blot. (B) Representative traces for I\textsubscript{SC} (top traces) and C\textsubscript{T} recordings (bottom) for control (black traces) and ENaC knockdown (grey traces) cells stimulated with forskolin (10 μM). (C) Summarized data for I\textsubscript{SC} versus C\textsubscript{T} responses to forskolin stimulation for control (n = 45) and βENaC (n = 55) knockdown cells similar to those presented in (B).

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supplementation compared to aldo supplemented cells at all time points and experimental conditions. For aldo supplemented cells, an increase in vesicle number was observed at 10 minutes compared to the 0 minute time point, and a significant increase in vesicle number was observed in cells stimulated by forskolin compared to unstimulated cells. This finding indicates a more rapid vesicle turnover in the presence of forskolin. In all cases, the number of vesicles per cell in unstimulated cells was significantly reduced compared to cells with supplementation. The data are in agreement with the electrophysiological recordings and support the idea that loss of ENaC expression results in a smaller vesicle pool that traffics to, and from, the apical surface.

**Discussion**

ENaC is the rate limiting step in Na⁺ absorption across epithelial tissues in which it is expressed. By altering the activity and density of ENaC in the apical membrane a precise regulation of Na⁺ absorption is achieved. Our previous work detailed the regulated recycling of ENaC at the apical membrane of kidney CCD cells (see Introduction). From the data presented above and our previous observations two distinct populations of trafficking and recycling ENaC can be observed in the mpkCCD cells. First, ENaC appears to be continuously recycled to and from the apical surface under non-stimulated steady-state conditions. Under basal/steady-state conditions, membrane capacitance is unchanged, however if the DUB responsible for removing ubiquitin from endocytosed ENaC is inhibited, an immediate rundown in ENaC-mediated I_sc was observed [17]. This observation suggested that while vesicles were still being delivered to the apical surface, ENaC that remained ubiquitylated was moved down a degradative path rather than being recycled. From data obtained in this study, the constitutive turnover of IgA was not impacted by alterations in ENaC expression; however a reduction in basal C_T was observed when ENaC expression was reduced. It is possible that ENaC may be recycled constitutively along with other apical surface proteins under unstimulated conditions, but the reduction in basal C_T would argue against this.

The population of ENaC that is rapidly recruited to the apical surface by exocytosis appears to be in a compartment that is dynamically regulated by cAMP agonists such as vasopressin or forskolin. An increase in ENaC abundance increases the number of vesicles trafficked and fused with the apical surface as determined using membrane capacitance. Stimulation of cAMP results in two responses. First there is a directed delivery of ENaC from a subapical source. Several lines of evidence point to a reserve population of ENaC that can traffic to the apical membrane in response to cAMP. The apical membrane capacitance increases in parallel with an increase in I_Na, indicating the exocytosis of ENaC-containing vesicles. Previous studies using biotin labeling demonstrated the delivery of additional channels to the surface following cAMP stimulation [37]. Coupled with this exocytic insertion event is acceleration in the turnover of apical membrane; that is, the rate of endocytosis is accelerated to match the increased rate of exocytosis. Evidence for this can be obtained from our previous study with the DUB inhibitor where the rate of I_sc rundown was accelerated in the presence of cAMP [17] and in this study where an increase in the number of endocytosed vesicles in the presence of cAMP stimulation is reported. The capacitance eventually reaches a steady state plateau after about 10 minutes of forskolin stimulation suggesting that the rate of endocytosis matches the accelerated exocytic rate. It is this rapidly mobilized vesicle pool that is regulated by ENaC abundance.

To understand the mechanisms that regulate this recycling compartment we considered at least two possibilities, namely that ENaC would be located in pre-existing recycling compartments and co-regulated with other apical transporters and proteins that are acutely trafficked in response to cAMP. Other apical channels and transporters are being trafficked in kidney epithelia and regulated concurrently with ENaC. In the principal cells of the distal kidney nephron, these include the water channel aquaporin 2 (Aqp2), the urea transporter (UT-A1) and possibly the potassium channel KCNJ1 or ROMK [38–70]. These transporters respond acutely to hormonal stimulation or intracellular signaling cascades.
in a manner similar to that described for ENaC. The action of vasopressin, acting through cAMP is known to induce the trafficking of Aqp2 and UT-A1 to the apical membrane by vesicle translocation [58]. If this trafficking response of multiple transporters to the same agonist involves the movement of vesicles from an intracellular store, as the time-courses suggest, then a fundamental question becomes how these transporters are differentially regulated.

A potential clue to answer this question can be found in the observations that the trafficking response to cAMP stimulation was never entirely eliminated. The number of trafficked vesicles was merely reduced with the loss in ENaC expression. In all cases where ENaC expression was altered an alternative trafficked vesicle pool remained intact. It is likely that these vesicles represent separate compartments involved in the trafficking of other membrane proteins, but this has yet to be determined.

Here we observed that ENaC trafficking and recycling was impacted by reducing ENaC's expression directly. The siRNA results indicate that the specific reduction of ENaC expression, without altering the aldosterone levels, produced the same reduction in the C_T response to cAMP stimulation as that observed in unsupplemented cells. This finding opens the possibility that a form of cargo recognition is occurring so that ENaC was able to recruit the necessary accessory proteins and trafficking partners required for its regulation, regardless of whether the expression levels of these accessory proteins change with aldosterone. There is precedent for this type of selective cargo-vesicle interaction [71–75]. A similar interaction between ion channel cargo and relative size of the cAMP induced exocytic events has been described for CFTR [76]. This regulatory complex would then be mediated by ENaC itself to allow for the selective trafficking of this channel independent from other transporters destined for the apical surface.

Recent reports have demonstrated differential regulation of ENaC with different cleavage states [77]. We altered the cleaved state of the channel to determine if this would alter the trafficking pathways or impact the vesicle-mediated CAMP response. In addition to investigating how cleavage may impact ENaC's regulation, we verified that the C_T recordings were not being altered by changes in Na^+ conduction through ENaC. While there was a clear reduction in ENaC conductance as recorded by the significant decrease in I_{Na}, the C_T response to cAMP stimulation was not significantly altered by the inhibition of ENaC activity. The addition of exogenous trypsin confirmed that ENaC was present in the apical membrane. Vesicle trafficking was not significantly impacted by preventing ENaC's proteolytic activation.

We present evidence that ENaC may be regulated in a unique fashion in epithelial cells. While the timing and trafficking kinetics induced by physiological cAMP agonists are similar for ENaC and other apically trafficked transporters, there appears to be a unique subset of vesicles that are responsible for the regulated trafficking of ENaC alone. This compartment adapts to accommodate ENaC numbers and is regulated separately from constitutive apical protein recycling. The presence of such a subset of vesicles allows for the differential regulation of transporters in response to different physiological cues. For example, in the kidney where coordinate trafficking of aquaporin and ENaC would be required to

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**Figure 5. ENaC expression in FRT cells.** (A) Representative I_{SC} trace from FTR epithelial cells transfected with αβγENaC (black trace) or control plasmid (grey trace), mounted in Ussing chambers and stimulated with 10 μM forskolin (grey bar). The expression of ENaC was confirmed by the addition of 10 μM amiloride (black bar) at the end of the recording. (B) Data from a number of similar experiments (n = 34) are summarized. Basal (-cAMP) and forskolin-stimulated (+cAMP) I_{Na} are presented on the left and the change in I_{SC} with forskolin stimulation (ΔI_{SC}) on the right (bar graph). (C) Representative capacitance trace from the same samples provided in (A). (D) Summarized data for capacitance changes under basal and forskolin-stimulated conditions as in (B). (* - indicates significantly different from untransfected p<0.05).

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allow for directional Na\(^+\) and water uptake, both transporters could be moved up to the apical surface in response to vasopressin [78–80]. Alternatively, if only one or two transporters are required it would offer cells the ability to discriminate the population of vesicles to be trafficked, and this disconnect in ENaC and AQP2 trafficking has been observed \textit{in vivo} [81]. The mechanisms behind such specialized trafficking events remain to be elucidated, and the regulation involved would therefore become increasingly important as we try and understand the cell biology that underlies the observed physiological response.

**Supporting Information**

**Figure S1** IgA recycling in mpkCCD cells. The percent IgA recycled over time for mpkCCD cells in fully supplemented media is provided. There is no significant difference for percentage IgA recycling at any time point.

**References**

1. Tahirovic S, Bradke F (2009) Neuronal polarity. Cold Spring HarbPerspectBiol 1: a001644.

2. Weisz OA, Rodriguez-Boulan E (2009) Apical trafficking in epithelial cells: signals, clusters and motors. Journal of Cell Science 122: 4253–4266.
3. Fohls H (2008) Regulation of membrane trafficking in polarized epithelial cells. Current Opinion in Cell Biology 20: 208–213.

20. McDonald FJ, Western AH, McNeil JD, Thomas BC, Olson DR, et al. (2002) The epithelial sodium channel (ENaC) traffics to apical membrane in lipid rafts in mouse cortical collecting duct cells. J Biol Chem 277: 43462–43471.

25. Wang H, Traub LM, Weixel KM, Hawryluk MJ, Shah N, et al. (2006) Clathrin-dependent ENaC trafficking requires the SNARE-binding protein complexin. Am J Physiol Renal Physiol 291: F196–F204.

28. Fakitsas P, Adam G, Daidie D, van Bemmelen MX, Fouladkou F, et al. (2007) Ubiquitin-protein ligase WWP2 binds to and downregulates the epithelial Na(+) channel by membrane trafficking. Biochimica et Biophysica Acta 1802: 1166–1177.

31. Mostov K, Apodaca G, Aroeti B, Okamoto C (1992) Plasma membrane protein sorting in polarized epithelial cells. J Biol Chem 267: 6271–6279.

34. Song W, Apodaca G, Mostov K (1994) Transcytosis of the polymeric immunoglobulin receptor is regulated in multiple intracellular compartments. J Biol Chem 269: 29474–29480.

35. Butterworth MB, Edlinger RS, Frizzell RA, Johnson JP, Edinger RS, et al. (2007) The epithelial sodium channel (ENaC) family and tissue distribution. J Biol Chem 282: 13753–13756.

37. Butterworth MB, Frizzell RA, Johnson JP, Peters KW, Edlinger RS (2005) PKA-dependent ENaC trafficking requires the SNARE-binding protein complexin. Am J Physiol Renal Physiol 290: F696–F707.

38. Leung SM, Ruiz WG, Apodaca G (2000) Sorting of membrane and fluid at the apical pole of polarized MDCK cells. Molecular Biology of the Cell in press.

39. Rojas R, Apodaca G (2002) Immune complex-induced ENaC transcytosis in primary cultures of rat cortical collecting duct cells. J Biol Chem 277: 22946–22950.

40. Vuagniaux G, Vallet V, Jaeger NF, Bens M, et al. (2000) Activation of the epithelial sodium channel by the serine protease mCAP1 is mediated by a signal transduction pathway requiring cAMP. J Biol Chem 275: 36586–36591.

41. Vuagniaux G, Vallet V, Jaeger NF, Rossier BC (1997) An epithelial Na(+) channel proteolytic cascade: routes to cell surface expression and regulation by proteolytic activity. J Biol Chem 272: 14553–14560.

42. Donaldson SH, Hirsh A, Li DC, Holloway G, Chao J, et al. (2002) Regulation of the Epithelial Sodium Channel in Neurophysiological and Hypertension 16: 444–450.

43. Knight KK, Wentzlaff DM, Snyder PM (2000) Intracellular sodium regulates prolyl-tRNA synthetase activity of the epithelial sodium channel. Journal of Biological Chemistry 275: 27477–27484.

44. Bruns JB, Carattino MD, Sheng S, Maarouf AB, Weisz OA, et al. (2007) Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. AJP - Renal Physiology 295: F190–F199.

45. Caldwell RA, Boucher RC, Stutts MJ (2004) Serine protease activation of near-silence epithelial Na(+) channels is regulated by mutations of the mouse cortical collecting duct cells. J Biol Chem 282: 37402–37411.

46. Lindstedt R, Apodaca G, Barondes SH, Mostov KE, Leffler H (1993) Apical secretion of a cytosolic protein by Madin-Darby canine kidney cells. Evidence for polarized release of an endogenous lectin by a nonclassical secretory pathway. J Biol Chem 268: 11750–11757.

47. Thompson M, Boucher RC, Perry JH, Wang H, Apodaca G, et al. (2002) The amiloride-sensitive epithelial Na(+) channel by the serine protease mCAP1 is expressed in a mouse cortical collecting duct cell line. J Am Soc Nephrol 11: 829–834.

48. Vaudry R, Grail D, Gaggello HP, Herzberger JD, Rossier BC (1997) An epithelial sodium channel protease activates the amiloride-sensitive sodium channel. Nature (London) 389: 607–610.

49. Kleyman TR, Myerburg MM, Hughey RP (2006) Regulation of ENaCs by proteases: An increasingly complex story. Kidney Int 70: 1391–1392.

50. Myerburg MM, Butterworth MB, McKenna EE, Peters KW, Frizzell RA, et al. (2006) An aorta- surface liquid ENaC is regulated by altered the serine-protease-protease inhibitor balance: a mechanism for sodium hyperabsorption in cystic fibrosis. J Biol Chem 281: 27942–27949.

51. Rosier BC (2004) The Epithelial Sodium Channel: Activation by Membrane- Bound Serine Proteases. Proceedings of the American Thoracic Society 1: 4–9.

52. Adelaburo A, Cheng Y, Johnson JP, Bridges RJ (2005) Endogenous protease activation of ENaC: effect of serum and thiol protease inhibition on ENaC single channel properties. J Gen Physiol 126: 339–352.

53. Caldwell RA, Boucher RC, Stutts MJ (2004) Neutrophil elastase activates near-silent epithelial Na+ channels and increases airway epithelial Na+ transport. Am J Physiol Lung Cell Mol Physiol 288: L813–L819.

54. Kleyman TR, Myrberg MM, Hugh RP (2006) Regulation of ENaCs by proteases: An increasingly complex story. Kidney Int 70: 1391–1392.

55. Myrberg MM, Butterworth MB, McKenna EE, Peters KW, Frizzell RA, et al. (2006) Airway surface liquid regulates ENaC by altering the serine protease-protease inhibitor balance: a mechanism for sodium hyperabsorption in cystic fibrosis. J Biol Chem 281: 27942–27949.

56. Rosier BC (2004) PAM2 activates epithelial Na+ channels by cleaving the gamma subunit. Journal of Biological Chemistry 280: 5123–5128.

57. Kleyman TR, Myrberg MM, Hugh RP (2006) Regulation of ENaCs by proteases: An increasingly complex story. Kidney Int 70: 1391–1392.

58. Myrberg MM, Butterworth MB, McKenna EE, Peters KW, Frizzell RA, et al. (2006) Airway surface liquid regulates ENaC by altering the serine protease-protease inhibitor balance: a mechanism for sodium hyperabsorption in cystic fibrosis. J Biol Chem 281: 27942–27949.

59. Rosier BC (2004) The Epithelial Sodium Channel: Activation by Membrane- Bound Serine Proteases. Proceedings of the American Thoracic Society 1: 4–9.

60. Adelaburo A, Cheng Y, Johnson JP, Bridges RJ (2005) Endogenous protease activation of ENaC: effect of serum and thiol protease inhibition on ENaC single channel properties. J Gen Physiol 126: 339–352.

61. Caldwell RA, Boucher RC, Stutts MJ (2004) Neutrophil elastase activates near-silent epithelial Na+ channels and increases airway epithelial Na+ transport. Am J Physiol Lung Cell Mol Physiol 288: L813–L819.
62. Noda Y, Sasaki S (2005) Trafficking mechanism of water channel aquaporin-2. BiolCell 97: 885-892.
63. Valenti G, Procino G, Tamma G, Carmosino M, Svelto M (2005) Mini-review: aquaporin 2 trafficking. Endocrinology 146: 5063–5070.
64. Ecelbarger CA, Kim GH, Mitchell GW, Terris J, Wade JB, et al. (1999) Regulation of the abundance of sodium and urea transporters along the nephron by vasopressin. PASEB J 13: A392.
65. Bagnasco SM (2005) Role and regulation of urea transporters. Pflugers Archiv 450: 217–228.
66. Sandi JM (2004) Renal urea transporters. CurrOpinNephrol Hypertens 13: 525–532.
67. Yoo D, Kim BY, Campo C, Nance L, King A, et al. (2003) Cell surface expression of the ROMK (Kir1.1) channel is regulated by the aldosterone-induced kinase, SGK-1, and protein kinase A. J BiolChem 278: 23066–23075.
68. Wang WH (2006) Regulation of ROMK (Kir1.1) channels: new mechanisms and aspects. AJP - Renal Physiology 290: F14–F19.
69. Wagner CA, Lotfiling-Cuenc DA, Yan Q, Schulz N, Fakinas P, et al. (2008) Mouse model of type II Bartter's syndrome. II. Altered expression of renal sodium- and water-transporting proteins. Am J Physiol Renal Physiol 294: F1373–1380.
70. Frindt G, Palmer LG (2010) Effects of dietary K on cell-surface expression of renal ion channels and transporters. Am J Physiol Renal Physiol 299: F890–897.
71. Shintani T, Klionsky DJ (2004) Cargo proteins facilitate the formation of transport vesicles in the cytoplasm to vacuole targeting pathway. J Biol Chem 279: 29089–29094.
72. Castle A, Castle D (2005) Ubiquitously expressed secretory carrier membrane proteins (SCAMPs) 1–4 mark different pathways and exhibit limited constitutive trafficking to and from the cell surface. J Cell Sci 118: 3769–3780.
73. Puthenveedu MA, von Zastrow M (2006) Cargo Regulates Clathrin-Coated Pit Dynamics. Cell 127: 113–124.
74. Leonard D, Hayakawa A, Lawe D, Lambright D, Bellve KD, et al. (2008) Sorting of EGF and transferrin at the plasma membrane and by cargo-specific signaling to EEA1-enriched endosomes. J Cell Sci 121: 3445–3458.
75. Yudowski GA, Puthenveedu MA, Henry AG, von ZM (2009) Cargo-mediated regulation of a rapid Rab4-dependent recycling pathway. Molecular Biology of the Cell 20: 2774–2784.
76. Bradbury NA, Jilling T, Berta G, Sorscher EJ, Bridges RJ, et al. (1992) Regulation of plasma membrane recycling by CFTR. Science 256: 330–332.
77. Knight KK, Olson DR, Zhou R, Snyder PM (2006) Liddle's syndrome mutations increase Na+ transport through dual effects on epithelial Na+ channel surface expression and proteolytic cleavage. ProcNatlAcadSciUSA 103: 2805–2808.
78. Ecelbarger CA, Kim GH, Wade JB, Knepper MA (2001) Regulation of the abundance of renal sodium transporters and channels by vasopressin. Exp Neurol 171: 227–234.
79. Ohara M, Cadnapaphornchai MA, Summer SN, Falk S, Yang J, et al. (2002) Effect of mineralocorticoid deficiency on ion and urea transporters and aquaporin water channels in the rat. Biochemical and Biophysical Research Communications 299: 285–290.
80. Frokiaer J, Nielsen S, Knepper MA (2005) Molecular Physiology of Renal Aquaporins and Sodium Transporters: Exciting Approaches to Understand Regulation of Renal Water Handling. Journal of the American Society of Nephrology 16: 2827–2829.
81. Wang W, Li C, Nejsum LN, Li H, Kim SW, et al. (2006) Biphasic effects of ANP infusion in conscious, euolemic rats: roles of AQP2 and ENaC trafficking. Am J Physiol Renal Physiol 290: F530–541.