Deubiquitylation RegulatesActivation and Proteolytic Cleavage of ENaC

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ABSTRACT

The epithelial sodium channel (ENaC) is critical for sodium and BP homeostasis. ENaC is regulated by Nedd4-2–mediated ubiquitylation, which leads to its internalization; this process can be reversed by deubiquitylation, which is regulated by the aldosterone-induced enzyme Usp2-45. In a second regulatory pathway, ENaC can be activated by luminal serine protease–mediated cleavage of its extracellular loops. Whether these two regulatory processes interact, however, is unknown. Here, in HEK293 cells stably transfected with ENaC, Usp2-45 interacted with ENaC, leading to deubiquitylation of the channel and stimulation of ENaC activity 20-fold. This was accompanied by a modest increase in cell surface expression of ENaC and by proteolytic cleavage of αENaC and γENaC at their extracellular loops. When endocytosis was inhibited with dominant negative dynamin (DynK44R), channel density and γENaC cleavage were increased, but αENaC cleavage and ENaC activity were not augmented. When Usp2-45 was coexpressed with DynK44R, both αENaC cleavage and activity were recovered. In summary, these data suggest that Usp2-45 deubiquitylation of ENaC enhances the proteolytic activation of both αENaC and γENaC, possibly by inducing a conformational change and by interfering with endocytosis, respectively.


The epithelial sodium channel (ENaC), composed of three subunits (α, β, and γ), is important for Na+ homeostasis and BP regulation.1 It is rate limiting in Na+ entry and tightly regulated by diverse mechanisms (including aldosterone). Of interest are two seemingly unrelated regulatory pathways, one involving the ubiquitin system, the other one luminal serine proteases. The first concerns the ubiquitin-protein ligase Nedd4-2 that interacts directly with ENaC, causing ubiquitylation and internalization of the channel.2–10 Ubiquitylation entails the linkage of ubiquitin to lysines on target proteins. This is achieved by an enzymatic cascade, including E1 and E2 enzymes and E3 ubiquitin-protein ligases.11 Deubiquitylation enzymes reverse the ubiquitylation level of target proteins.12 Recently it was shown by Fakitsas et al.6 that aldosterone induces the expression of the deubiquitylation enzyme Usp2-45 in the cortical collecting duct. They demonstrated in Xenopus laevis oocytes that Usp2-45 stimulates amiloride-sensitive Na+ currents and that Usp2-45 deubiquitylates α and γENaC. The other mechanism of ENaC regulation implicates the action of serine proteases and is fundamentally different from the one involving ubiquitin, because it acts either in the lumen of the secretory pathway or extracellularly.13,14 Thereby, the proteases modulate ENaC by cleaving the extracellular loop of either α or γENaC.15–19 Little is known about the regulation of this process, but it seems to involve aldosterone, as evidenced by the observation that in mice or rats...
kept under low-Na\(^+\) diet or treated with aldosterone, \(\gamma\)ENaC seems to be cleaved as well.\(^{19,21}\) Here we provide evidence that the two regulatory mechanisms are related to each other in that the degree of ubiquitylation controlled by the balance of Nedd4-2 and Usp2-45 regulates the level of cleaved ENaC at the cell surface by a multistep mechanism in which aldosterone, via induction of Usp2-45 protein, stimulates ENaC deubiquitylation, leading to the accumulation of cleaved \(\alpha\) and \(\gamma\)ENaC at the plasma membrane.

**RESULTS**

**HEK293 Cells Stably Expressing ENaC Display Small Amiloride-Sensitive Na\(^+\) Currents**

Previously we showed that Usp2-45 increases ENaC activity when coexpressed in *Xenopus laevis* oocytes.\(^7\) Such an increase could be due to a change of intrinsic channel properties, an increase of channel number at the cell surface, or a combination of the two. To study this question, we generated stable HEK293 cell lines expressing all three ENaC subunits. Expression of \(\alpha\)ENaC, tagged with a triple HA epitope at its C-terminus,\(^22\) was under the control of a glucocorticoid-inducible promoter.\(^23\) \(\beta\)ENaC tagged with myc and \(\gamma\)ENaC with vesicular stomatitis virus (VSV) tag were expressed from a constitutive cytomegalovirus promoter. Representative Western blots against the tags demonstrated that all three subunits are expressed (\(\alpha\)ENaC at 100 and 72 kD; \(\beta\)ENaC at 100 kD, and \(\gamma\)ENaC at 95 kD), and the expression of \(\alpha\)ENaC was under tight control of dexamethasone (Figure 1A). Blotting with ENaC antibodies revealed endogenous, cross-reacting proteins (Figure 1B; \(\alpha\) and \(\gamma\)ENaC); however, we were unable to detect by real-time PCR mRNA encoding \(\alpha\), \(\beta\), and \(\gamma\)ENaC in untransfected HEK293 cells. Moreover, these endogenous proteins were not sensitive to deglycosylation of PNGase F, as would be expected due to an increase of intrinsic channel properties, an increase of channel number at the cell surface, or a combination of the two. To study this question, we generated stable HEK293 cell lines expressing all three ENaC subunits. Expression of \(\alpha\)ENaC, tagged with a triple HA epitope at its C-terminus,\(^22\) was under the control of a glucocorticoid-inducible promoter.\(^23\) \(\beta\)ENaC tagged with myc and \(\gamma\)ENaC with vesicular stomatitis virus (VSV) tag were expressed from a constitutive cytomegalovirus promoter. Representative Western blots against the tags demonstrated that all three subunits are expressed (\(\alpha\)ENaC at 100 and 72 kD; \(\beta\)ENaC at 100 kD, and \(\gamma\)ENaC at 95 kD), and the expression of \(\alpha\)ENaC was under tight control of dexamethasone (Figure 1A). Blotting with ENaC antibodies revealed endogenous, cross-reacting proteins (Figure 1B; \(\alpha\) and \(\gamma\)ENaC); however, we were unable to detect by real-time PCR mRNA encoding \(\alpha\), \(\beta\), and \(\gamma\)ENaC in untransfected HEK293 cells. Moreover, these endogenous proteins were not sensitive to deglycosylation of PNGase F, as would be expected due to a change of intrinsic channel properties, an increase of channel number at the cell surface, or a combination of the two.

**Usp2-45 Stimulates the Expression of Functional ENaC Channels by Deubiquitylating ENaC**

We transfected these cells with either wild-type or inactive Usp2-45 (Usp2-45-C67A) and repeated the whole-cell patch-clamp measurements (Figure 2A). Expression of Usp2-45 increased current densities by \(>20\)-fold and required the active enzyme. The current-voltage relationship of the induced current exhibited a reversal potential of the current at +55 mV, consistent with a Na\(^+\)-selective current (Figure 2B). We determined the Na\(^+\)/K\(^+\) permeability ratio of this current to be 151 ± 47 (n = 10) from the shift in the current-voltage relationship, when exchanging the extracellular monovalent cation from Na\(^+\) to K\(^+\) (Figure 2C). The current was inhibited by amiloride with an IC\(_{50}\) of 94 ± 3 nM (n = 12; Figure 2D). The functional properties identified the current induced by overexpression of Usp2-45 as mediated by ENaC.

To follow ubiquitylation of ENaC at the cell surface, we biotinylated the HEK293 cells expressing ENaC, recovered the channel subunits by immunoprecipitation, and pulled down the biotinylated fraction of the immunoprecipitated material with streptavidin Sepharose. This material was immunoblotted with either ENaC or ubiquitin antibodies (Figure 3). Biotinylated ENaC subunits were detectable at the expected sizes (Figure 3, bottom). To detect the signal for ubiquitylated ENaC subunits, we coexpressed dominant negative dynamin (DynK44R), an inhibitor of endocytosis (Figure 3, lane 6). Blotting with anti-ubiquitin antibodies in this condition revealed discrete bands for \(\alpha\) (125 kD), \(\beta\) (130 kD), and \(\gamma\)ENaC (120 kD), suggesting that at the cell surface, ENaC subunits exist as ubiquitylated species with a defined number of ubiquitin moieties, in contrast to the total pool of channel subunits in the cell lysate, which is mostly polyubiquitylated (Supplemental Figure S1 and ref. 6). When Usp2-45 was co-trans-
Usp2-45 and Nedd4-2 regulate channel density at the plasma membrane. We made another set of cell surface biotinylation experiments and purified the biotinylated proteins by pull-down with streptavidin Sepharose. This material was analyzed down with streptavidin Sepharose. This material was analyzed down with streptavidin Sepharose. This material was analyzed by SDS-PAGE/immunoblotting, using ENaC antibodies. Indeed, the data in Figure 3 tend to suggest that Usp2-45 and Ned4-2 regulate channel density at the plasma membrane. We made another set of cell surface biotinylation experiments and purified the biotinylated proteins by pull-down with streptavidin Sepharose. This material was analyzed by SDS-PAGE/immunoblotting, using ENaC antibodies. Figure 4A shows that the lysates and nonbiotinylated fraction of α and βENaC subunits were not affected by the expression of Usp2-45 or its mutant. In contrast, we observed weak, faster migrating species for γENaC (with apparent molecular weight between 73 and 77 kD) when Usp2-45 was co-transfected (Figure 4A, middle and top, asterisk). Consistent with the notion that ubiquitylation controls cell surface expression of ENaC, the amount of the ENaC subunits increased significantly at the cell surface when Usp2-45 was coexpressed (Figure 4A, bottom). Quantification of three different experiments indicated roughly a three-fold increase for α and approximately two-fold for β and γENaC (Figure 4B). Importantly, the majority of γENaC changed its apparent molecular weight and migrated as a doublet (Figure 4A, biotinylated fraction), which was estimated on a high-resolution gel to migrate at 77 and 73 kD (Supplemental Figure S2). Similarly, we observed for αENaC that Usp2-45 induced an approximately 100-kD form, a prominent 28-kD protein, and minor 29- and 32-kD products (Supplemental Figure S3, bottom, long exposure, blotted with αENaC antibodies recognizing the N-terminus). Occasionally, we also saw the appearance of fragments at approximately 70 kD. In contrast, blotting with anti-HA (recognizing the C-terminus) revealed a doublet at 66 and 71 kD. We were not able to detect the 100-kD protein with the HA antibody. Similar changes have been described previously for both α and γENaC and attributed to the activity of luminal serine proteases, such as furin, prostaclin, or elastase.15,18-20,26,27 To confirm that the observed bands for γENaC represent proteolytic products, we mutated γENaC in either the furin site (rat 135-RKR→138) or the protasin site 178-RKR→181 to QQQQ (R178→181Q) described previously.17,26 Cells were transiently transfected with αβENaC, wild-type or mutated γENaC, and active or inactive Usp2-45, and cell surface biotinylation was carried out. Figure 5 (biotinylated fraction) shows that the mutation of the furin (lane 6) but not the protasin site (lane 9) abolished the main 77-kD fragment, corroborating the idea that this was a furin-dependent cleavage product. Conversely, in this condition, the 73-kD fragment was not affected by either mutation; hence, the nature of this protein fragment remains to be clarified. Our data suggest that Usp2-45 causes a
γENaC Cell Surface Expression and the Biochemical Properties of γENaC

Our previous data suggested that the balance of Usp2-45 and Nedd4-2 activities may control the cell surface pool of active ENaC. We therefore transfected HEK293-αβγENaC cells with varying ratios of Nedd4-2 and Usp2-45 and examined both α and γENaC in the whole-cell lysate, non-biotinylated and biotinylated fractions (Figure 6). The ENaC subunits were not significantly affected in the total lysate, and in the non-biotinylated fraction; however, in the biotinylated cell surface fraction, αENaC was less prominent, relative to the control, when Nedd4-2 was expressed, and increased with escalating proportions of Usp2-45. Similarly, the amount of proteolytic fragments of γENaC increased with higher Usp2-45 expression. Hence, Usp2-45 counteracts Nedd4-2–induced ubiquitylation and increases the ENaC pool at the cell surface as well as the cleavage of γENaC.

Usp2-45 Controls Cleavage of α and γENaC via Different Modes of Action

There are at least two ways in which Usp2-45 may regulate cleavage of α and γENaC. Deubiquitylation of ENaC could induce a conformational alteration rendering the proteolytic site accessible, or it may interfere with endocytosis, thereby slowing down the turnover of ENaC and increasing the probability of a cleavage. To test the second model, we interfered with endocytosis by co-transfecting dominant negative dynamin (DynK44R),

Deubiquitylating Enzyme Usp2-45 Interacts with ENaC

To understand how Usp2-45 regulates ENaC activity, we tested whether Usp2-45 interacts with the ENaC channel complex. As can be seen in Supplemental Figures S4 and S5, Usp2-45 interacted via its C-terminal region with ENaC. Moreover, this interaction did not involve the PY motifs of ENaC (Supplemental Figure S6). We also show that endogenous Usp2-45 is co-immunoprecipitated with endogenous αENaC in renal epithelial mCCD1,1 cells (Supplemental Figure S7).

>20-fold increase of amiloride-sensitive Na⁺ currents that is accompanied by two- to three-fold augmentation of cell surface expression of the ENaC subunits and increased cleavage of both α and γENaC. (the latter at the furin site and possibly at another cleavage site yet to be defined).

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with DynK44R restores αENaC cleavage and activity, as deubiquitylated channels accumulate at the plasma membrane. This prediction was indeed confirmed as shown in Figures 7B and 9. No significant difference between ENaC + Usp2-45 or ENaC + Usp2-45 + DynK44R was observed with respect to αENaC cleavage (Figure 7B), cell surface expression as evidenced by biotinylation (Figure 9A), or amiloride-sensitive currents (Figure 9B). These data therefore suggest that deubiquitylation controls proteolytic activation of ENaC, possibly by inducing a conformational change in the case of αENaC or by interfering with internalization (γENaC).

**DISCUSSION**

It is well established that ubiquitylation of ENaC controls cell surface expression of the channel and that this regulation is defective in Liddle syndrome, a salt-sensitive form of hypertension; however, ubiquitylation of ENaC is a reversible process, involving aldosterone-induced Usp2-45. Here, we studied the molecular mechanisms of Usp2-45-dependent regulation and present evidence that Usp2-45 regulates ENaC in a multistep process: (1) It binds to and deubiquitylates ENaC, (2) this deubiquitylation inhibits ENaC internalization and activates ENaC by inducing cleavage of αENaC, and (3) Inhibition of channel endocytosis favors cleavage γENaC.

The presented data reinforce the idea that the ubiquitin system constitutes a powerful system to regulate ENaC activity and consequently protects against excess Na\(^+\) influx. Indeed, HEK293 cells stably transfected with αβγENaC subunits expressed significant levels of the three subunits (Figure 1) but displayed low amiloride-sensitive Na\(^+\) currents (Figure 2). The co-transfection of Usp2-45 elevated these currents by >20-fold, with properties (K\(_t\) amiloride, reversal potential, Na\(^+\) over K\(^+\) selectivity) corresponding to the ones documented for ENaC channels. This was accompanied by the deubiquitylation of the ENaC subunits (Figure 3 and Supplemental Figure S1). Interestingly, the deubiquitylation by Usp2-45 not only increased the number of channels at the plasma membrane but...
Ergonul et al. 90-kD/H9253 indicates the 77-kD band; arrowhead indicates the 73-kD fragment. Experiments were carried out as described in Figure 4. Asterisk units. Comparable observations were made by Knight et al., who showed that Liddle mutations (interfering with Nedd4-2–dependent ubiquitylation) increased cell surface expression, and cleavage of ENaC.

Changes in the apparent molecular weight of the α and γ subunits were reported previously. Masilamani et al., who described that salt restriction caused up-regulation of α and β subunits and an increase of a 75-kD γENaC protein in rat kidneys. This was also reported by Ergonul et al., who described that salt restriction caused up-regulation of α and β subunits and an increase of a 75-kD γENaC fragment, accompanied by a decrease in the full-length 90-kD γENaC. Evidence was provided by several laboratories that both α and γENaC were cleaved by proteases. It was shown that the cleavage of αENaC occurred at two consensus sites for furin, generating 65- and 30-kD fragments and the one of γENaC at cleavage sites for furin and prostasin, yielding a 65-kD polypeptide (for a detailed review of ENaC proteolysis, see references13). We now partly confirm these findings by showing that both αENaC and γENaC are cleaved and, by mutating the furin site on γENaC (R138A), demonstrating that the generation of the 77-kD fragment depends on this site; however, we do not know the nature of the additional 73-kD fragment in γENaC, because mutation of neither the furin nor the prostasin site alters the appearance of this band.

Figure 5. Usp2-45 induces furin-dependent cleavage of γENaC. HEK293 cells were transiently transfected with α and βENaC, together with WT γENaC, or R138A or R178-181Q mutated γENaC and with Usp2-45 (WT or inactive mutant) as indicated. Experiments were carried out as described in Figure 4. Asterisk indicates the 77-kD band; arrowhead indicates the 73-kD fragment.

Figure 6. The balance between Usp2-45 and Nedd4-2 regulates the cell surface expression of ENaC. Stably transfected HEK293 cells (α, β, and γENaC) were transfected with a total of 20 μg of DNA (comprising different ratios of plasmids encoding Usp2-45 or Nedd4-2: 1, pcDNA3; 2, 1 μg of Usp2-45; 3, 0.75 μg of Usp2-45 + 0.25 μg of Nedd4-2; 4, 0.5 μg of Usp2-45 + 0.5 μg of Nedd4-2; 5, 0.25 μg of Usp2-45 + 0.75 μg of Nedd4-2; 6, 1 μg of Nedd4-2). Whole-cell lysate and nonbiotinylated and biotinylated fractions were recovered as in Figure 4 and analyzed for α and γENaC by SDS-PAGE/Western blotting with the indicated antibodies.
induce cleavage of γENaC, suggesting that it is the prolonged stay of ENaC at the cell surface that favors this cleavage event, dominant negative dynamin does not promote cleavage of αENaC. This therefore suggests that deubiquitylation triggers an event that likely involves conformational changes within the channel complex rendering the proteolytic sites on αENaC accessible.

The findings that dominant negative dynamin does not increase ENaC activity are different from published ones, in which DynK44R stimulated ENaC activity. A possible explanation for this may be that HEK293 cells display much stronger Nedd4-2–dependent inhibition, as evidenced by the observation that Usp2-45 has to be expressed to measure decent ENaC currents (Figure 2), whereas this is not necessary in CHO cells or oocytes. Our findings confirm reports by others that the accumulation of ENaC at the cell surface and cleavage of γENaC are not sufficient for proper ENaC activation. Most likely, Usp2-45–dependent deubiquitylation of ENaC or another protein involved in ENaC regulation, promoting the cleavage of αENaC, is as important as to activate the Na⁺ channel. Indeed, co-expression of Usp2-45 with DynK44R restores ENaC activity at the cell surface without affecting expression levels or cleavage of γENaC (Figure 9). In addition, a mutant ENaC channel, in which all cytoplasmic lysines (i.e., putative ubiquitylation sites) are mutated to arginine, cannot be further stimulated with Usp2-45 (both functionally and biochemically by cleavage6 (D.R.-D. and O.S., unpublished observations). Such a scenario seems to variance with published data that suggest that ubiquitylation of ENaC controls the cell surface expression and not its intrinsic properties; however, these differences may be explained again by the different cellular system used in these studies (Xenopus laevis oocytes). Conversely, ENaC channels with Liddle mutations (mutations of PY motifs and hence missing the binding site for the ubiquitin–protein ligase Nedd4-2) display not only increased channel number at the membrane but also higher intrinsic activity. What is the physiologic relevance of these findings? Usp2-45, which is an aldosterone-induced protein, may well represent the link that controls the observed aldosterone-induced cleavage of α and γENaC in vivo. Thereby, aldosterone induces Usp2-45 expression, which binds to ENaC and counteracts Nedd4-2 action by deubiquitylating ENaC. The deubiquitylation of the channel will increase open probability (possibly by promoting a conformational change and rendering accessible an αENaC cleavage site) and enhance channel density, either by interfering with its internalization or by enhancing recycling, thereby favoring γENaC cleavage (Figure 10).

CONCISE METHODS

DNA Constructs
All ENaC constructs were based on rat ENaC. Dexamethasone-inducible plkneo-αENaC-(HA)₃ was described previously. Rat βENaC was
tagged with a c-myc tag and γENaC with a VSV tag, both at the C-terminus, and cloned into pcDNA3. For binding experiments, a γENaC construct containing a Flag tag on its extracellular loop (as described previously43) and cloned into pCB6 was used. The ENaC PY mutants were previously described6 and subcloned into pcDNA3. The ENaC lysine-to-arginine mutants were generated by PCR as described previously6 and subcloned in pcDNA3.1(…). The ENaC furin mutant R138A and ENaC prostasin mutant R178–181Q were generated using site-directed mutagenesis and subcloned in pcDNA3.1(…). Usp2-45 wild type and C67A were generated by PCR as described previously and cloned into pcDNA3.6 Usp2-45 N-terminal amino acids 1 through 53 and Usp2-45 C-terminal amino acids 53 through 342 were labeled with an S tag at the N-terminal side and generated by PCR. Usp2-45 N-terminal was subcloned into a pCMV GST vector, and Usp2-45 C-terminal was subcloned as the full length of Usp2-45 in pcDNA3.1(…). The dynamin constructs were previously described28 and provided by S. Schmid (Scripps Research Institute, La Jolla, CA) and subcloned into pcDNA3.

Generation of Stable HEK293 Cells Expressing α, β, and γENaC

HEK293 cells stably expressing rat HA-tagged α, myc-tagged β, and VSV-tagged γENaC were generated. C-terminally triple HA-tagged

αENaC in pLKneo was described before22,23 and contains a dexamethasone-inducible promoter and a G418 resistance gene. C-terminally tagged βENaC was cloned into pCEP4 (containing a hygromycin re-
Aldosterone-induced Usp2-45 interacts with the channel complex and deubiquitylates it, thereby allowing a conformational change in ENaC and rendering accessible the cleavage sites of αENaC. Deubiquitylation also prolongs the stay at the cell surface leading to γENaC cleavage.

**Figure 10.** Proposed mechanism of ENaC regulation by Nedd4-2, Usp2-45, and proteolytic cleavage. Nedd4-2 ubiquitylates ENaC on all three subunits and promotes internalization and lysosomal degradation of ENaC. Aldosterone-induced Usp2-45 interacts with the channel complex and deubiquitylates it, thereby allowing a conformational change in ENaC and rendering accessible the cleavage sites of αENaC. Deubiquitylation also prolongs the stay at the cell surface leading to γENaC cleavage.

**Cell Surface Biotinylation**

Cells were washed 24 h after transfection, twice with cold PBS (1×), then treated for 30 min with 4 ml biotin (0.3 mg/ml; EZ link Sulfo-NHS-SS-Biotin; Pierce, Rockford, IL) per 10-cm dish at 4°C. They were washed twice with cold TBS (1×) and lysed in 1-ml/dish lysis buffer (as described in previous section). The cells were solubilized for 3 h on a wheel at 4°C and centrifuged for 30 min at 20,000 × g at 4°C. Supernatants were recovered, and protein concentrations were quantified by the Bradford method (Coo protein dosage kit; Interchim, Montluçon, France). Small parts of the lysates were kept to verify the transfection efficiency. Thirty microliters of streptavidin Sepharose beads was added to 0.7 to 2.0 mg of total proteins and incubated overnight on a wheel at 4°C. A small part of the supernatants was recovered to have the nonbiotinylated fraction. The beads were washed five times with lysis buffer, and dried beads were resuspended in 30 μl of 100 mM dithiothreitol. They were incubated for 1 h at 37°C with shaking. Then they were centrifuged for 2 min at 11,000 × g to recover the supernatant. Sample buffer (5×; 1.47 M sucrose, 10% SDS, 5 mM EDTA, 300 mM Tris [pH 8.8], 0.25% Bromophenol blue, and 130 mM dithiothreitol) was added, and the proteins were run on SDS-PAGE, transferred onto nitrocellulose, and analyzed by Western blotting.

**Determination of Ubiquitylation of ENaC Subunits at the Cell Surface**

Cells expressing ENaC were biotinylated as described in the previous section. Then they were lysed in 500 µl of lysis buffer as described in the previous section. Cells were vortexed for 15 s and centrifuged for 15 min at 20,000 × g at 4°C. Immunoprecipitation was carried out as described in the previous section, except that proteins were not eluted with sample buffer. Instead, dried beads were incubated with 100 µl of PBS (1×) and 1% SDS and boiled for 15 min at 99°C under agitation. Supernatants were recovered, and 900 µl of PBS (1×) was added together with 30 µl of streptavidin Sepharose beads (GE Healthcare, Buckinghamshire, UK). They were incubated for 1 h on a rotating wheel at 4°C. Beads were then washed two times with 1× PBS/1% Triton X100. Dried beads were resuspended in 30 µl of 2× sample buffer. The samples were run on SDS-PAGE, transferred onto nitrocellulose, and analyzed by Western blot, using antiubiquitin antibodies (FK2; Biomol International, Plymouth Meeting, PA).

**Immunoprecipitation from Transfected HEK293 Cells**

Cells were dissociated 24 h after transfection in 1 ml of cell dissociation buffer (5% glycerol, 1 mM EDTA, and 1 mM EGTA in PBS). Then the cells were recovered with 10 ml of PBS (1×) in 15 ml of polypropylene tubes. After 2 min of centrifugation at 3000 × g, supernatants were discarded, and the pellets were frozen at −70°C for at least 20 min. They were lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10% glycerol, and 1% Triton X100) containing proteases inhibitors (protease inhibitor cocktail complete; Roche, Basel, Switzerland), 0.2 mM N-ethylmaleimide (Fluka, Buchs, Switzerland), 1 mM dithiothreitol (Sigma), and phosphatases inhib-
itors (100 mM NaF and 10 mM Di-Na-pyrophosphate) in a 1.5-ml tube. Cells were solubilized for 30 min on a wheel at 4°C. The cells were then centrifuged for 15 min at 20,000 × g. Supernatants were recovered, and protein concentrations were quantified by the Bradford method. Immunoprecipitations were performed with a minimum of 400 μg of total proteins. 1/250 of antibody (HA [Santa Cruz Biotechnology, Santa Cruz, CA] for αENaC, c-Myc [Sigma] for βENaC, flag M2-Agarose [Sigma] for γENaC, biotinylated S-Protein [Novagen, Madison, WI] for Usp2-45, or anti-Usp2 for endogenous Usp2) was added to the lysate and incubated for 2.5 h on a rotating wheel at 4°C. Then 20 μl of Protein G Sepharose (GE Healthcare; for HA, c-myc), Protein A Sepharose (GE Healthcare; for Usp2-45), or streptavidin Sepharose (GE Healthcare; for S-protein) was added to the mix protein-antibody and incubated in the same conditions during 1.25 h. Four washes were done with 1 ml of wash buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EGTA, 10% glycerol, 0.2% Triton X100, and 1.5 mM MgCl2), the centrifugations were done at 3000 × g. Dried beads were resuspended in 40 μl of sample buffer (2X). Then the samples were run on SDS-PAGE, transferred onto nitrocellulose, and analyzed by Western blot.

Antibodies/Western Blotting

Anti αENaC antibodies were raised against the N-terminus and the β and γ antibodies against the C-terminus, as described previously, and used at a dilution of 1:500. Tagged αENaC was also detected with anti-HA antibodies (1:500; Santa Cruz Biotechnology), βENaC with anti c-myc (1:500; Sigma), and γENaC either with anti–Flag-M2 antibody (Sigma; in transient transfection for binding studies) or with anti-VSV antibody (1:500; Sigma; in all of the other experiments). Anti–Usp2-45 antibodies were generated against GST-Usp2-45 (amino acids 1 through 49) and used at a dilution of 1:500. Tagged Usp2-45 was also detected with commercial S-protein horseradish peroxidase (1:10,000; Novagen). Anti–Nedd4-2 (Abcam, Cambridge, UK) was diluted 1:500 for immunoblots. Anti-actin (Sigma) was used (in transient transfection for binding studies) or with anti-HA antibodies (1:500; Santa Cruz Biotechnology), anti-c-myc antibodies against the C-terminus, as described previously, and used at a dilution of 1:500 for endogenous Nedd4 antibodies.

Electrophysiologic Measurements and Analysis

Electrophysiologic measurements were taken at 24 to 48 h after induction of the αENaC subunit. Macromolecular amiloride-sensitive currents, defined as the difference between ionic currents obtained in the absence and presence of 10 μM amiloride in the bath, were recorded using the patch-clamp technique in the whole-cell configuration at room temperature. All macromolecular currents shown are amiloride-sensitive currents as defined above in this paragraph. We used an EPC-10 amplifier (HEKA Electronics, Lambrecht, Germany) and Pulse and PulseFit software for data acquisition and analysis. The sampling interval was 5 ms for all measurements except for current-voltage relationships, for which it was 100 μs. Filtering was set to 5 kHz. For rapid changes of extracellular solutions, we used a micro manifold that brings eight tubes into one outlet tube (MPRE8; Cell MicroControls, Norfolk, VA). The solution flow was controlled by computer-driven solenoid valves. The standard extracellular solution contained 140 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM MES, 10 mM HEPES, and 10 mM glucose, and pH was adjusted to 7.4 with NaOH. For selectivity measurements, we used solutions containing 10 mM MES, 10 mM HEPES, and 10 mM glucose with either 140 mM NaCl or 140 mM KCl. pH was adjusted to 7.4 with Tris-base. Pipettes were pulled from Borosilicate glass (World Precision Instruments, Sarasota, FL). Pipettes had a resistance of 1 to 3 MΩ when filled with the pipette solution. The pipette solution contained 90 mM K gluconate, 10 mM KCl, 10 mM NaCl, 1 mM MgCl2, 60 mM HEPES, and 10 mM EGTA (pH 7.3 adjusted with KOH). 

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