AMP-activated Kinase Inhibits the Epithelial Na\(^+\) Channel through Functional Regulation of the Ubiquitin Ligase Nedd4-2*

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We recently found that the metabolic sensor AMP-activated kinase (AMPK) inhibits the epithelial Na\(^+\) channel (ENaC) through decreased plasma membrane ENaC expression, an effect requiring the presence of a binding motif in the cytoplasmic tail of the β-ENaC subunit for the ubiquitin ligase Nedd4-2. To further examine the role of Nedd4-2 in the regulation of ENaC by AMPK, we studied the effects of AMPK activation on ENaC currents in Xenopus oocytes co-expressing ENaC and wild-type (WT) or mutant forms of Nedd4-2. ENaC inhibition by AMPK was preserved in oocytes expressing WT Nedd4-2 but blocked in oocytes expressing either a dominant-negative (DN) or constitutively active (CA) Nedd4-2 mutant, suggesting that AMPK-dependent modulation of Nedd4-2 function is involved. Similar experiments utilizing WT or mutant forms of the serum- and glucocorticoid-regulated kinase (SGK1), modulators of protein kinase A (PKA), or extracellular-regulated kinase (ERK) did not affect ENaC inhibition by AMPK, suggesting that these pathways known to modulate the Nedd4-2-ENaC interaction are not responsible. AMPK-dependent phosphorylation of Nedd4-2 expressed in HEK-293 cells occurred both in vitro and in vivo, suggesting a potential mechanism for modulation of Nedd4-2 and thus cellular ENaC activity. Moreover, cellular AMPK activation significantly enhanced the interaction of the β-ENaC subunit with Nedd4-2, as measured by co-immunoprecipitation assays in HEK-293 cells. In summary, these results suggest a novel mechanism for ENaC regulation in which AMPK promotes ENaC-Nedd4-2 interaction, thereby inhibiting ENaC by increasing Nedd4-2-dependent ENaC retrieval from the plasma membrane. AMPK-dependent ENaC inhibition may limit cellular Na\(^+\) loading under conditions of metabolic stress when AMPK becomes activated.

The epithelial Na\(^+\) channel (ENaC)\(^2\) is an apical Na\(^+\) channel expressed in a variety of salt-reabsorbing epithelial tissues, including the kidney, lung, exocrine gland ducts, and colon (1). This channel plays a major role in the regulation of total body salt and volume homeostasis, blood pressure, and airway surface liquid clearance. ENaC is comprised of three structurally similar α-, β-, and γ-subunits, whose full-length forms are \(~90–95\) kDa in size. Each subunit has two presumed transmembrane domains, a large extracellular loop, and cytoplasmic N and C termini (1). ENaC expressed in oocytes has a presumed αβγ stoichiometry (2, 3), although alternate subunit stoichiometries have been proposed (4, 5). ENaC can be identified by its sensitivity to the diuretic amiloride, its ohmic current-voltage relationship, and its high selectivity for conductance of Na\(^+\) and Li\(^+\) over K\(^+\) (1).

ENaC is regulated by the actions of several hormones, including aldosterone, vasopressin, and insulin, as well as various non-hormonal mechanisms. Cellular mechanisms that control ENaC activity include the regulation of channel synthesis, intracellular trafficking, membrane insertion and retrieval, and single channel properties (1). In addition, recent work suggests that intracellular cleavage of ENaC α- and γ-subunits in the biogenesis pathway by proteases such as furin (forming active subunits of \(~65\) and \(~75\) kDa, respectively) plays a critical role in the regulation of cellular ENaC activity (6–8).

Several signaling pathways have been identified that are involved in ENaC regulation, including kinases such as PKA, protein kinase C, SGK, the phosphoinositide (PI) 3-kinase pathway, and the MAPK/ERK signaling cascades (9–14). Of note, the interaction between the PY motifs on the C termini of β- and γ-ENaC and the WW domains of Nedd4-2, an E3 ubiquitin-protein ligase, has emerged as a critical locus for ENaC activity regulation by these signaling pathways (13, 15–17).

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‡ The abbreviations used are: ENaC, epithelial Na\(^+\) channel; AMPK, AMP-activated protein kinase; WT, wild-type; DN, dominant-negative; CA, constitutively active; SGK, serum- and glucocorticoid-regulated kinase; PKA, cAMP-dependent protein kinase; ERK, extracellular-regulated kinase; ZMP, 5-aminoimidazole-4-carboxamide-1-β-D-ribosanovyl S'-monophosphate; BFA, brefeldin A; HRP, horseradish peroxidase; xNedd4-2, Xenopus Nedd4-2; mSGK1, mouse SGK1; mENaC, mouse ENaC; HA, hemagglutinin; cRNA, complementary RNA; SSOs, supplemented standard oocyte solution; TEV, two-electrode voltage clamp; Tet, tetracycline; co-IP, co-immunoprecipitation; CFTR, cystic fibrosis transmembrane conductance regulator; ANOVA, analysis of variance; PI, phosphoinositide; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase.
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Nedd4-2-mediated ubiquitination of ENaC promotes ENaC internalization from the plasma membrane and degradation. SGK1 is stimulated through aldosterone-induced transcription or through activation of the PI 3-kinase pathway. SGK1 phosphorylates Nedd4-2 at two key residues, Ser-338 and Ser-444 in Xenopus Nedd4-2 (xNedd4-2) (18). This phosphorylation disrupts the ENaC-Nedd4-2 interaction, apparently by enhancing the binding affinity of Nedd4-2 to 14-3-3 proteins, which sequester Nedd4-2 from ENaC, as we and others (19, 20) have recently shown. Disruption of the ENaC-Nedd4-2 interaction by naturally occurring gain-of-function mutations involving the PY motif in the ENaC tail can cause Liddle’s syndrome, a disease characterized by hypertension, volume expansion, and hypokalemia (21). The associated reduction in ENaC ubiquitination increases ENaC stability and expression at the plasma membrane. PKA also phosphorylates Nedd4-2 at the same residues to inhibit ENaC internalization and may also promote increased delivery and insertion of ENaC into the plasma membrane (14). ERK may directly phosphorylate the C termini of β- and γ-ENaC and promote Nedd4-2-mediated ENaC retrieval (13).

It has long been recognized that inhibition of cellular metabolism through depletion of metabolic substrates (22–24) or hypoxia (25, 26) inhibits apical epithelial Na+ channels, as well as other transport proteins (27, 28). However, the underlying mechanisms involved in metabolic depletion-associated transport inhibition are unclear. In recent years, the metabolic sensor AMPK has emerged as a potential mediator for transport-metabolism coupling, as this kinase has been shown to regulate nutrient transporters and Cl− and Na+ channels (28).

AMPK is a ubiquitous metabolic-sensing Ser/Thr kinase that exists as a heterotrimer composed of a catalytic α-subunit and regulatory β- and γ-subunits. Its activity increases during conditions of cellular metabolic stress, in response to elevated intracellular AMP:ATP ratios (29), and in response to other upstream signaling kinases (30, 31). AMPK appears to act as a key sensor of cellular energy homeostasis that oppositely regulates energy-utilizing and energy-generating metabolic pathways. In addition, AMPK has been implicated in the regulation of a rapidly growing list of other important cellular functions, including cell growth, apoptosis, inflammation, gene transcription, and protein synthesis (32, 33). Indeed, AMPK may modulate several important kinase-dependent cellular signaling pathways, including the MAPK/ERK and PKC signaling pathways known to regulate ENaC (34–36).

We recently found that AMPK inhibits ENaC in both oocytes and mouse polarized kidney epithelial cells (37). AMPK-dependent regulation of ENaC has also been subsequently shown in polarized lung epithelial cells (38). This regulation appears to be indirect, as AMPK did not bind or phosphorylate ENaC in vitro. AMPK inhibited ENaC expression at the plasma membrane in oocytes. This inhibition was blocked with expression of a β-ENaC mutant that disrupts the binding of Nedd4-2 to ENaC, suggesting that Nedd4-2 plays a central role in the ENaC inhibition by AMPK (37).

The aims of this study were to further investigate the mechanism of ENaC regulation by AMPK and characterize the role of Nedd4-2 in this regulation. Our findings suggest that AMPK activation enhances the rate of ENaC retrieval from the plasma membrane and that Nedd4-2 plays a pivotal role in the AMPK-dependent inhibition of ENaC. Nedd4-2 also serves as a target for AMPK-dependent phosphorylation. Moreover, cellular AMPK activation enhances the association of Nedd4-2 and ENaC, thereby providing a plausible mechanism for enhanced ENaC turnover at the plasma membrane and inhibition by AMPK.  

EXPERIMENTAL PROCEDURES

Reagents and Chemicals—All chemicals used were obtained from Sigma unless otherwise noted. ZMP (5-aminomidazole-4-carboxamide-1-β-d-ribofuranosyl 5’-monophosphate) was obtained from Toronto Research Chemicals (North York, Ont., Canada). Brefeldin A (BFA) was obtained from Tocris Bioscience (Ellisville, MO). U0126 was obtained from Promega (Madison, WI). Anti-hemagglutinin antibody (HA.11) was obtained from Covance (Princeton, NJ), Anti-V5 and horseradish peroxidase (HRP)-conjugated anti-V5 antibodies were obtained from Invitrogen. Secondary HRP-conjugated antimouse IgG antibody was obtained from Amersham Biosciences (Piscataway, NJ). Plasmids for mammalian or oocyte expression of WT and mutant N-terminal, FLAG-tagged Xenopus Nedd4-2 (FLAG-xNedd4-2), Mouse SGK1 (mSGK1), and mouse ENaC (mENaC) subunits were generated and used as described previously (20, 37). Purified recombinant active human AMPK holoenzyme (α1-T172D, β1, γ1) and inactive AMPK holoenzyme (α1-D157A, β1, γ1) were synthesized in Escherichia coli using a tricistronic plasmid and purified as described (39).

Oocyte Expression—Maintenance of Xenopus laevis frogs, surgical extraction of ovaries, and collagenase treatment of oocytes were carried out as described (40). Complementary RNAs (cRNAs) for all proteins expressed in oocytes were synthesized using the mMessage mMachine kit (Ambion, Austin, TX). The purity and quantity of cRNA were assessed by agarose gel electrophoresis. Stage V-VI oocytes were injected with cRNAs within 1 day of collagenase treatment. Oocytes were then maintained in supplemented standard oocyte solution (sSOS) containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4 supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamycin at 18 °C for 1–2 days before experimentation.

Two-electrode Voltage Clamp—Two-electrode voltage clamp (TEV) measurements were performed using oocytes continuously perfused with TEV solution containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl2, and 10 mM HEPES, pH 7.4, as described previously (37). To determine current-voltage relationships, a series of 500-ms voltage steps from −140 to +60 mV in 20-mV increments were performed. Currents were routinely measured after 400 ms at a clamp potential of −100 mV. ENaC-mediated Na+ currents were defined as the current difference measured in the absence versus the presence of 10 μMamiloride in the bath solution. To activate cellular AMPK for experiments, 40 mM potassium-ZMP (K-ZMP) or potassium-glucanate (K-glucanate; control) was microinjected into oocytes (32 nl/oocyte) ≥1 h prior to TEV measurements (37).
Generation and Characterization of HEK-293 Cells Stably Expressing Tetracycline-inducible AMPK Mutants—N-terminal, HA-tagged rat AMPK-α1 (WT) or AMPK-α1-K45R (DN) cDNA was subcloned by PCR from pTracer-HA-AMPK-α1 or -α1-K45R into the KpnI and XhoI sites of the pcDNA5/FRT/TO vector (Invitrogen) to stably introduce these genes into the genome of HEK-293 Flp-In TREX® cells under the control of a tetracycline (Tet)-inducible promoter. All plasmids were verified by DNA sequencing. Transient transfection and selection of stable transfectants was performed according to the manufacturer’s recommendations. Verification of Tet-inducible expression of the transgenes was confirmed by immunoblotting for the HA-tagged protein in cell lysates. Cellular AMPK activity was measured using the SAMS peptide in vitro kinase assay, which was performed on immunoprecipitates of AMPK from cell lysates using AMPK-α anti-serum (generous gift of Lee Witters), as described previously (41, 42).

Cell Culture and Transfection—HEK-293 Flp-In TREX® cells expressing either Tet-inducible WT- or DN-AMPK were maintained in Dulbecco’s Modified Eagle’s Medium (BioWhittaker) supplemented with 4.5 g/liter glucose and 1-glutamine, 10% Tet System-approved fetal bovine serum (Clontech), appropriate selection antibiotics (15 μg/ml blasticidin, 150 μg/ml hygromycin), and penicillin/streptomycin, and passaged twice weekly. Cells were seeded at subconfluent density into 60-mm dishes 3 days prior to phosphorylation and co-immunoprecipitation (co-IP) experiments and induced as appropriate with 1 μg/ml Tet in the medium. Transfection was performed using Lipofectamine 2000 (Invitrogen) and 5–6 μg of total plasmid DNA per transfection according to the manufacturer’s recommendations.

Immunoblotting of Oocyte Lysates—Lysis and immunoblotting of oocytes for total ERK1/2 and activated phospho-ERK1/2 expression was performed as described previously (43), except that a 4–12% gradient gel (NuPAGE, Invitrogen) was used for SDS-PAGE.

In Vitro Phosphorylation Assays—Uninduced HEK-293 cells transiently transfected to express FLAG-xNedd4-2 or human CFTR were lysed 1 or 2 days after transfection, respectively. Nedd4-2 or CFTR was immunoprecipitated from cell lysates using the M2 anti-FLAG monoclonal antibody or the m24–1 anti-CFTR monoclonal antibody coupled to protein A/G beads (Pierce), as described (20, 37). In vitro phosphorylation was performed using purified active or inactive AMPK holoenzyme with [γ-32P]ATP labeling, as described (37). After SDS-PAGE and transfer to nitrocellulose membranes, immunoblotting for expression of FLAG-xNedd4-2 or CFTR was first performed and quantitated using a Versa-Doc Imager with Quantity One software (Bio-Rad). After the chemiluminescent signal had decayed, phosphorylated bands on the membrane were imaged by exposure to a phosphoscreen, and the bands were quantitated using a Bio-Rad Phosphorimager. The intensity of each band was corrected by subtracting out the local background in the same lane.

In Vivo Phosphorylation Assays—Either uninduced or Tet-induced, stably transfected HEK-293 Flp-In TREX® cells expressing WT- or DN-AMPK were transiently transfected with the pMO-FLAG-xNedd4-2 plasmid (20) 1 day prior to experimentation. For labeling, cells were washed twice in phosphate-free eflux buffer containing (in mM): 140 NaCl, 3 KCl, 1 MgSO4, 1 CaCl2, 10 glucose, and 10 HEPES (pH 7.4 with 1 M Tris base). Each dish of cells was then incubated at 37 °C for 5 h in 1.5 ml of this buffer containing 0.3 mCi of [32P]orthophosphate (MP Biomedicals, Inc., Irvine, CA) and 2–5 mM metformin, an AMPK activator (44), to further activate AMPK in the uninduced and Tet-induced WT-AMPK-expressing cells during this labeling period. After this incubation, cells were washed once in ice-cold phosphate-free buffer before lysis in ice-cold radioimmunoprecipitation assay buffer containing (in mM): 50 Tris-HCl, pH 7.5, 100 NaCl, 50 NaF, 1 EDTA, 1 EGTA, 1 phenylmethylsulfonyl fluoride, 1 sodium orthovanadate, 0.1 mg/ml aprotinin, 1 complete protease inhibitor mixture (Roche Applied Science) 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100 (phosphatase and protease inhibitors added just prior to use). After clearing the lysates by high speed centrifugation, FLAG-xNedd4-2 was immunoprecipitated, washed, eluted in Laemmlli sample buffer, subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane. As described above, Nedd4-2 immunoblotting was performed first followed by phosphorimager analysis of the same membrane. The ratio of the phosphorylation signal to the immunoblot signal in each lane was compared across conditions to derive relative phosphorylation levels.

ENaC and Nedd4-2 Co-immunoprecipitations from HEK-293 Cells—Tet-induced WT-AMPK or DN-AMPK-expressing HEK-293 cells were co-transfected to express C-terminal epitope-tagged α-, β-, and γ-mENaC (6) with or without FLAG-xNedd4-2 1 d prior to experimentation using 2 μg of plasmid DNA for α- and γ-mENaC and 1 μg of plasmid DNA for β-ENaC and FLAG-xNedd4-2 per transfection. Only one of the three mENaC subunits had a V5 epitope tag for each transfection; the other epitope tags were hemagglutinin (HA) for α- and β- and c-Myc for γ-ENaC. After treatment for 5 h with 5 mM metformin, cells were lysed in ice-cold lysis buffer phosphate-buffered saline containing 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1× complete protease inhibitor mixture, and insoluble material was pelleted out by centrifugation at 13,000 × g. After quantitating the protein concentration using the Bradford assay (Bio-Rad), a 75-μg sample from each cell lysate was removed for SDS-PAGE prior to immunoprecipitation (IP). IPs were performed overnight at 4 °C on each lysate sample containing 1.5 mg of total protein using either the M2 anti-FLAG antibody (0.5 μg/IP) or anti-V5 monoclonal antibody (1 μg/IP; Invitrogen) coupled to protein A/G beads. After three washes in lysis buffer, the IP sample was then eluted in Laemmlli sample buffer and, along with the cell lysate samples, subjected to SDS-PAGE. Immunoblotting was performed at 1:2500 dilution in 5% bovine serum albumin using hors eradish peroxidase-conjugated anti-V5 (Invitrogen) or anti-FLAG antibodies to compare the amount of protein co-precipitated relative to its expression in the cell lysates. Membranes were then reprobed at 1:7500 with the other antibody to detect the amount of immunoprecipitated protein for each condition. Relative binding was quantitied by dividing the co-IP protein signal by the signal for that protein in the cell lysate.
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which was then corrected for the amount of immunoprecipitated protein for that condition. For each experimental condition, the binding measured with AMPK-activated (WT-AMPK + metformin) was quantitated and expressed relative to that with AMPK-inhibited (DN-AMPK + metformin).

Statistical Analysis—TEV data generated from different oocyte batches were pooled, and statistical analyses were performed using an ANOVA factorial model to account for batch-to-batch variability in amiloride-sensitive ENaC currents. In the figure legends, n indicates the number of oocytes measured, and N indicates the number of oocyte batches used. For other biochemical experiments, statistics were performed using unpaired or paired Student’s t tests, as described for each figure. StatView (SAS Institute Inc., Cary, NC) was used for statistical analyses. In all cases, p values < 0.05 were considered significant.

RESULTS

We recently found that AMPK inhibits ENaC expressed in Xenopus oocytes and in polarized renal collecting duct cells that endogenously express the channel (37). This inhibition appeared to occur through reduced expression of active channels at the plasma membrane and was abrogated with expression of a Liddle’s syndrome-type β-mENaC mutant that precludes binding of Nedd4-2 to the tail (37, 45). These findings suggest that AMPK may regulate ENaC via Nedd4-2 activation, enhancing Nedd4-2-dependent retrieval of ENaC from the plasma membrane.

AMPK Enhances ENaC Retrieval from the Plasma Membrane—To test whether AMPK does enhance endocytic retrieval of ENaC from the plasma membrane, we measured changes in ENaC currents in the presence or absence of brefeldin- A (BFA) as a function of AMPK activation. BFA is a fungal toxin that inhibits the secretion of newly synthesized proteins to the plasma membrane by disassembly and redistribution of the Golgi network into the endoplasmic reticulum (46). Treatment of oocytes with BFA inhibits anterograde trafficking of channels to the plasma membrane and thus offers an approach to examine rates of channel retrieval (47, 48). We have previously shown that injection of the AMPK activator ZMP (an AMP analogue) into oocytes inhibits mENaC currents in an AMPK-dependent fashion (37). If AMPK stimulates ENaC retrieval from the plasma membrane, a greater reduction of ENaC currents is expected after BFA treatment in K-ZMP-injected versus potassium gluconate (control)-injected oocytes. Representative current-voltage plots obtained by TEV recordings on oocytes with or without a prior 3-h exposure to BFA are shown in Fig. 1A. K-ZMP-injected oocytes had significantly decreased ENaC currents compared with potassium gluconate-injected controls, both in the absence (Fig. 1B, left) and presence (Fig. 1B, right) of BFA, suggesting that the AMPK-dependent inhibition of ENaC is mediated by enhanced ENaC retrieval from the plasma membrane. If decreased delivery of new channels were primarily responsible for the ENaC inhibition, no significant difference in ENaC currents would be expected after BFA treatment.

AMPK-dependent Inhibition of ENaC Involves Modulation of Nedd4-2 Function—Our previous work utilizing a β-mENaC Y618A mutant subunit suggested that ENaC inhibition by AMPK requires the binding of Nedd4-2 to ENaC (37). To further explore the potential role of Nedd4-2 in this inhibition, we measured amiloride-sensitive currents in oocytes co-expressing mENaC and either WT or mutant xNedd4-2 constructs (Fig. 2). Co-expression of WT xNedd4-2 substantially reduced ENaC currents relative to those observed with expression of ENaC alone (not shown), but AMPK activation through K-ZMP injection into oocytes still inhibited ENaC current (Fig. 2, A and B, left), as found previously with expression of ENaC.

FIGURE 1. Activation of AMPK inhibits ENaC currents by enhanced retrieval of channels from the plasma membrane. A, representative current-voltage plots of amiloride-sensitive ENaC currents recorded by TEV. Pretreatment TEV measurements were obtained in a batch of oocytes expressing mENaC and either WT or mutant xNedd4-2 constructs. These injected oocytes were then incubated in sSOS containing 5 μM BFA or Vehicle Control (0.05% Me2SO) for 3 h prior to TEV measurements. Current values at –100 mV were used for comparison purposes across conditions (dashed line). B, relative ENaC currents after treatment with BFA or vehicle control with (K-ZMP) or without (K-gluc) AMPK activation. All values were normalized to the mean pretreatment current for each experiment (dashed line at ±1. *p < 0.05 relative to K-gluc paired controls; †, p < 0.01 relative to vehicle control; ANOVA; n = 27–32 oocytes, N = 4 batches). Oocytes were injected with 1 ng/subunit of mENaC cRNAs for each experiment.
alone (37). Co-expression of a ubiquitin ligase-deficient, DN xNedd4-2 mutant (C938S; Ref. 49) greatly increased ENaC currents (Fig. 2A), presumably by preventing Nedd4-2-dependent ENaC ubiquitination and degradation. However, under this condition K-ZMP failed to inhibit ENaC (Fig. 2B), suggesting that Nedd4-2-mediated ENaC ubiquitination is necessary for the AMPK-dependent inhibition. Conversely, co-expression of a CA xNedd4-2 mutant (S338A, S444A) lacking two dominant SGK1/PKA phosphorylation sites that promote binding of Nedd4-2 to 14-3-3 proteins (and presumed Nedd4-2 sequestration from ENaC) (19, 20), substantially inhibited ENaC current (Fig. 2A, right). Again, K-ZMP failed to inhibit ENaC current with co-expression of this xNedd4-2 mutant (Fig. 2B, right). Taken together, these findings indicate that modulation of Nedd4-2 activity is necessary for the AMPK-dependent inhibition of ENaC.

ENaC Inhibition by AMPK Does Not Require Modulation of SGK1 Activity—AMPK-dependent modulation of the Nedd4-2 regulation of ENaC could potentially occur through specific effects on Nedd4-2 function, through an upstream regulator of Nedd4-2, such as SGK1 or PKA (or their upstream signaling regulators), or through effects on ENaC that promote its association with Nedd4-2 (e.g. through regulation of kinases that phosphorylate the C-tail of ENaC, such as ERK) (13, 49). To explore the potential role of SGK1 in the inhibition of ENaC by AMPK, we have performed similar TEV measurements utilizing WT-, DN- (K127M), and CA- (S422D) mSGK1 mutants. Mean (±S.E.) of absolute ENaC currents measured in oocytes 1–3 h after injection of K-gluc or K-ZMP (A) and ENaC currents normalized to the mean of K-gluc-injected oocytes for each individual experiment (B) are shown (*, p < 0.001 relative to K-gluc control, ANOVA, n = 49–51 oocytes, N = 8 batches). Oocytes were injected with 1–3 ng/subunit of mENaC and 5 ng of xNedd4-2 cRNAs for each experiment.

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FIGURE 4. ENaC currents as a function of AMPK activation with stimulation of cellular PKA activity. Oocytes expressing mENaC and xNedd4-2 were injected with K-gluc or K-ZMP and then treated with PKA stimulation mixture (1 mM IBMX, 10 μM forskolin, and 200 μM cAMP) or vehicle control (0.2% Me2SO) 1 h later. TEV measurements of ENaC currents were then performed within 2.5 h after PKA stimulation. Values shown are the mean ENaC currents (±S.E.) for each condition normalized to the mean currents for K-gluc-injected vehicle controls within the same experiment (*, p < 0.005 relative to K-gluc paired controls; ‡, p < 0.001 relative to K-gluc vehicle controls, ANOVA; n = 20–22 oocytes, N = 3 batches). Oocytes were injected with 2.5–3.5 ng/subunit of mENaC and 2.5–5 ng of WT-xNedd4-2 cRNAs for each experiment.

PKA stimulation. As the PKA stimulation mixture did not override AMPK-dependent ENaC inhibition, these findings suggest that AMPK does not inhibit ENaC via inhibition of PKA activity.

MAPK/ERK Activation Cannot Account for AMPK-dependent ENaC Inhibition—Activation of the MAPK/ERK pathway may enhance ENaC endocytosis (10, 13), and it has been reported that AMPK may regulate this pathway (35, 36, 51). To test whether activation of this pathway is required for the AMPK-dependent inhibition of ENaC in oocytes, we used U0126, an inhibitor of MEK (the upstream kinase that activates ERK), to block signaling through the MAPK/ERK pathway. ENaC-expressing oocytes were pretreated with U0126 (or vehicle control) and then injected with K-ZMP (to activate AMPK) or potassium gluconate (control) prior to TEV measurements of ENaC currents (Fig. 5A). U0126 treatment did not significantly affect baseline ENaC currents in potassium gluconate-injected oocytes, consistent with the recent observation that this pathway is normally quiescent in oocytes under resting conditions (43). Of note, ENaC current was similarly inhibited by K-ZMP in the presence or absence of U0126 treatment (Fig. 5A), suggesting that ERK activation is not required for the AMPK-dependent inhibition. Similar results were obtained using the MEK inhibitor PD98059 (not shown). As further controls for these studies, we performed immunoblots on lysates from ENaC-expressing, potassium gluconate versus K-ZMP injected oocytes that were preincubated for 6–8 h in media with or without 6 μM progesterone to activate the ERK pathway in the oocytes and/or U0126 to block this activation (Fig. 5B). The activated form of ERK was detected using a specific phospho-ERK1/2 antibody (upper panel), as described previously (43). After stripping the membrane, it was reprobed to detect total ERK (lower panel). No significant ERK activity was detectable under resting conditions in untreated potassium gluconate-injected oocytes, as evidenced by the lack of a phospho-ERK band in lane 3. AMPK activation by K-ZMP injection slightly activated ERK (lane 4), but this ERK activation was blocked by treatment with U0126 (lane 2). As expected, progesterone treatment alone activated ERK substantially (lanes 5 and 6), but again this activation was completely blocked in the presence of U0126 (lanes 7 and 8). Together, these results suggest that although AMPK may activate ERK to some extent in this system, which could play a small role in the inhibition of ENaC, this activation is not sufficient to account for the full AMPK-dependent ENaC inhibition, as this inhibition was unaffected by U0126 treatment, which fully suppressed any detectable ERK activation.

AMPK Phosphorylates Nedd4-2 in Vitro—As the regulation of Nedd4-2 has been implicated in AMPK-dependent ENaC inhibition, we considered that Nedd4-2 could serve as a target for AMPK phosphorylation. To test this possibility, we per-
formed in vitro phosphorylation experiments of FLAG-tagged xNedd4-2 expressed in and then immunoprecipitated from HEK-293 cell lysates (Fig. 6). As found for the positive control CFTR (lower panels), but not for ENaC (37) or β-actin (not shown), purified active but not inactive AMPK holoenzyme phosphorylated full-length Nedd4-2 in vitro (upper panels). These findings suggest that AMPK might directly modulate Nedd4-2 function (and thus ENaC membrane expression) through Nedd4-2 phosphorylation.

Modulation of Intracellular AMPK Activity—For in vivo phosphorylation and binding studies, we have developed HEK-293 cells that are stably transfected with either HA-tagged WT-AMPK-α1 or DN-AMPK under a Tet-inducible promoter. Uninduced cells do not express detectable HA-tagged AMPK protein, and up to 3 days of Tet induction is optimal for protein expression (Fig. 7A). Suppression of cellular AMPK activity, as measured by an in vitro kinase assay performed on cell lysates, was maximal at 3 days after Tet induction (data not shown). There was a trend toward cellular AMPK activation in uninduced cells by a 5-h treatment with the AMPK activator metformin (Fig. 7B, left) (44). This activation was completely blocked in Tet-induced cells over-expressing DN-AMPK (Fig. 7B, center). Moreover, the Tet-induced DN-AMPK-expressing cells have significantly inhibited AMPK activity relative to that of uninduced controls and Tet-induced WT-AMPK-expressing cells (Fig. 7B). Thus, to effectively modulate AMPK activity for in vivo phosphorylation and binding assays, we have compared results obtained in metformin-treated, Tet-induced DN-AMPK-expressing cells (AMPK-inhibited) with either metformin-treated, Tet-induced WT-AMPK expressing cells or uninduced cells (AMPK-activated).

AMPK-dependent Phosphorylation of Nedd4-2 in Vivo—To determine whether AMPK-dependent Nedd4-2 phosphorylation occurs in vivo, we have compared intracellular [γ-32P]orthophosphate labeling of Nedd4-2 under AMPK-activated versus AMPK-inhibited conditions (Fig. 8). Nedd4-2 immunoblotting was performed first followed by phosphorimager analysis of the same membrane (Fig. 8A). The ratio of the phosphorylation signal to the immunoblot signal in each lane was compared across conditions to derive relative phosphorylation levels (Fig. 8B). Relative to AMPK-inhibited cells, Nedd4-2 phosphate labeling significantly increased by ∼50% in AMPK-activated cells (44 ± 11% in metformin-treated WT-AMPK-expressing cells and 54 ± 21% in metformin-treated uninduced cells (Fig. 8B)). These results indicate that significant AMPK-dependent phosphorylation of Nedd4-2 occurs in vivo. Background phosphorylation in the absence of AMPK activation may represent baseline Nedd4-2 phosphorylation by other cellular kinases (e.g. PKA and SGK).

AMPK Activity-dependent Modulation of the ENaC-Nedd4-2 Interaction—The results presented so far suggest that AMPK inhibits ENaC through activation of Nedd4-2-dependent ENaC retrieval from the plasma membrane. To test whether this mechanism involves an AMPK-dependent enhancement in the binding of ENaC to Nedd4-2, FLAG-xNedd4-2 was co-

![Image](324x492 to 552x733)

**FIGURE 6.** AMPK-dependent phosphorylation of Nedd4-2 occurs in vitro. FLAG-tagged xNedd4-2 (top) or CFTR (positive control for AMPK phosphorylation; bottom), transiently expressed in HEK-293 cells and then immunoprecipitated from cell lysates, was subjected to in vitro phosphorylation using [γ-32P]ATP and purified active (A) or inactive (I) AMPK holoenzyme, as described under "Experimental Procedures." Representative phosphoscreen images (upper panels) and immunoblots (lower panels) of the same membrane are shown, along with results from parallel experiments performed on mock-transfected HEK-293 cells (right).

**FIGURE 7.** Tet-induced expression of HA-tagged WT- or DN-AMPK-α1 modulates cellular AMPK activity in stably transfected HEK-293 cells. A, immunoblot demonstrating WT- (left) and DN- (right) HA-tagged AMPK-α1 expression at various days after Tet-induction. B, mean (± S.E.) AMPK activity by SAMS peptide in vitro kinase assay in uninduced cells (left), 3 day-induced DN-AMPK-α1-expressing cells (center), and 1 day-induced WT-AMPK-α1-expressing cells (right) ± 2–5 mM metformin treatment for 5 h (†, p = 0.10 and *, p < 0.001 compared with uninduced Con; unpaired Student's t-tests, n = 4–15 experiments).
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As shown in Fig. 10, the association of β-mENaC with xNedd4-2 was significantly increased with AMPK activation relative to that with AMPK inhibited, as assessed by co-IPs in both directions (by $\sim 125 \pm 50\%$ for IP of V5-β-ENaC and probe for FLAG-xNedd4-2, $\sim 75 \pm 25\%$ for IP of FLAG-xNedd4-2 and probe for the mature form of V5-β-ENaC, and $\sim 55 \pm 20\%$ for IP of FLAG-xNedd4-2 and probe for the immature form of V5-β-ENaC). No significant AMPK-dependent differences in the apparent affinity for the binding of α- or γ-ENaC to Nedd4-2 were detected in these assays. There were also no apparent AMPK-dependent differences in the relative binding of cleaved or mature versus uncleaved or immature forms of the channel subunits to Nedd4-2. Taken together, our findings indicate that the AMPK-dependent inhibition of ENaC may be mediated through an AMPK-dependent enhancement in the apparent affinity of Nedd4-2 for β-ENaC, which enhances Nedd4-2-dependent ENaC retrieval from the plasma membrane and subsequent degradation.

DISCUSSION

Previous investigations have demonstrated that disruption of cellular metabolism can inhibit ion transport through apical channels such as ENaC (22–26). As a key regulator of cellular energy homeostasis, AMPK is a plausible candidate for the transduction of signals produced by depletion of metabolic substrates and hypoxia into a reduction in $\text{Na}^+$ transport through ENaC. Our recent work suggested that AMPK is a novel regulator of ENaC activity both in oocytes and in polarized epithelial cells (37). AMPK inhibited ENaC currents even in the presence of a degenerin site mutation that locked open channel gating, consistent with an effect on cell surface expression rather than open probability (37). In addition, AMPK did not appear to directly interact with or phosphorylate ENaC. Furthermore, AMPK-dependent inhibition of ENaC current was blocked in the presence of a Liddle’s-type mutation (β-mENaC-Y618A), arguing for a role of the C-terminal tail of β-ENaC in the regulation by AMPK and suggesting that binding of the E3 ubiquitin ligase Nedd4-2 is required (37).

Nedd4-2 has been shown to inhibit ENaC in both cultured cells and heterologous expression systems (52, 53). There are several lines of evidence to suggest that Nedd4-2 interacts with the β- and γ-subunits of ENaC through their C-terminal tails and that this interaction is important for reduction of ENaC cell surface expression (49, 53). Recent work has also suggested that Nedd4-2 may affect open probability in addition to an effect on cell surface expression (54, 55). Ubiquitination of ENaC is also known to promote retrieval of the channel from the plasma membrane (56). Our earlier results (37), along with our current finding suggesting that AMPK activation enhances ENaC retrieval from the plasma membrane (Fig. 1), directed us to the hypothesis that AMPK inhibits ENaC through effects on Nedd4-2.

We have demonstrated that co-expression of Nedd4-2 does not disrupt the inhibition of ENaC by AMPK, but inactive Nedd4-2 abolishes the AMPK effect on ENaC (Fig. 2). While the MAPK/ERK pathway is known to negatively regulate ENaC through the C-terminal tails of β- and/or γ-subunits (13), and AMPK can activate the MAPK/ERK pathway in other systems expressed with α-, β-, and γ-ENaC (with a C-terminal V5 epitope tag on one of the three subunits) in metformin-treated, WT- or DN-AMPK-expressing HEK-293 cells. Reciprocal co-IPs using anti-FLAG and anti-V5 antibodies were then performed and quantitated to assess the relative binding of each ENaC subunit to Nedd4-2 under AMPK-activated versus AMPK-inhibited conditions (Fig. 9). Both uncleaved and mature, cleaved forms of α- and γ-ENaC were apparent (Fig. 9, A and C), as well as immature and mature β-ENaC (Fig. 9B). Little nonspecific binding was observed in the co-IPs performed on cells expressing only epitope-tagged ENaC subunits without FLAG-xNedd4-2 (third and fourth lanes of each blot). Of note, there was an enhancement in the ratio of cleaved or mature forms of ENaC subunits to uncleaved or immature forms in the co-precipitating fraction as compared with that ratio in the whole cell lysates (compare co-IP lanes with cell lysate lanes in upper blots of each panel). This result is consistent with the idea that Nedd4-2 predominantly associates with a processed ENaC pool in the cell.
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(35, 36, 51), this mechanism does not appear to account for the majority of ENaC inhibition by AMPK (Fig. 5). Additionally, AMPK-dependent phosphorylation of Nedd4-2 occurs in vitro and in vivo (Figs. 6 and 8). Finally, activation of AMPK enhances the association of Nedd4-2 with β-ENaC in cells (Figs. 9 and 10), providing a general plausible mechanism for the inhibition of ENaC by AMPK.

The specific mechanisms by which AMPK alters the ability of Nedd4-2 to interact with ENaC remain unresolved. Direct phosphorylation is one mechanism by which ubiquitin ligases can be regulated. SGK1 and/or PKA are known to negatively regulate ubiquitin ligases by phosphorylation and induction of an inhibitory interaction with 14-3-3 proteins (19, 20). This interaction has been suggested to disrupt the Nedd4-2-ENaC interaction or inhibit the ligase activity of Nedd4-2 (57, 58). Phosphorylation of the E3 ubiquitin ligase Itch, another Nedd4 family member, by c-Jun N-terminal kinase (JNK) has been recently shown to disrupt an inhibitory intramolecular interaction between the catalytic HECT domain and an upstream interaction site, inducing a conformational change and activating the ligase (59). AMPK appears to enhance the interaction of Nedd4-2 with its apparent substrate, ENaC, but further characterization of this mechanism will be important. Nedd4-2 phosphorylation by AMPK could potentially disrupt the Nedd4-2-14-3-3 interaction that sequesters Nedd4-2 from ENaC. AMPK appears to be ineffective in inhibiting ENaC currents in the presence of a Nedd4-2 construct (S338A/S444A) where two known SGK1/PKA phosphorylation/14-3-3 interaction sites are mutated. An AMPK-dependent conformational change within Nedd4-2 that stabilizes the interaction between the WW domains of Nedd4-2 and the PY motif of β-ENaC is also possible. AMPK phosphorylation could also potentially recruit an accessory protein that stabilizes the interaction between Nedd4-2 and β-ENaC.

Although cellular AMPK activation enhanced the β-ENaC-Nedd4-2 interaction, no significant AMPK-dependent differences in the binding of α- or γ-ENaC to Nedd4-2 were found (Fig. 10). The simplest interpretation of this somewhat unexpected result is that AMPK primarily regulates Nedd4-2 interaction with the β-subunit in the channel complex and that α and γ may not remain as well associated with the complex after cellular lysis and during subsequent washes of the immunopre-

FIGURE 9. Reciprocal co-immunoprecipitations of epitope-tagged mENaC subunits and xNedd4-2 from HEK-293 cell lysates with cellular AMPK either activated or inhibited. IPs were performed with either anti-FLAG antibody (upper blots in each panel) or anti-V5 antibody (lower blots in each panel), and then membranes were probed using the other antibody to determine the amounts of co-precipitated protein (left lanes) relative to protein expression in the cell lysate (5% of IP input; right lanes). The membranes were then reprobed with the antibody used for IP (not shown) to correct for any differences in the amount of immunoprecipitated protein across conditions, so that relative binding could be quantitated (cf. Fig. 10). Representative immunoblots from at least four reciprocal co-IPs are shown for V5-tagged (A) α-ENaC, (B) β-ENaC, and (C) γ-ENaC with FLAG-N4-2. Cleaved and uncleaved α- and γ-ENaC bands and mature glycosylated and immature β-ENaC bands are identified. The intermediate band(s) between the uncleaved and cleaved forms of α-ENaC (A) may represent unglycosylated, uncleaved α-ENaC (55).
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FIGURE 10. AMPK activation enhances cellular interaction of Nedd4-2 with β-ENaC subunit. Results shown are the mean (±S.E.) apparent ENaC-Nedd4-2 binding relative to that with AMPK inhibited in each experiment (dashed line at 1). Results are shown for co-IPs in both directions and for both the cleaved and uncleaved bands of α- and γ-ENaC. Densitometric quantitation of the relevant bands and determinations of relative binding strength were performed as described under “Experimental Procedures.” Numbers of experiments for each condition are shown (*, p < 0.05; compared with the AMPK-inhibited condition, paired Student’s t-test).

precipitate. However, alternate channel stoichiometries and non-coordinate subunit regulation are additional intriguing possibilities that can be envisioned. In addition, as AMPK does not appear to phosphorylate β-ENaC directly (37), it is conceivable that AMPK-dependent regulation of the activity of other kinases that phosphorylate β-ENaC and modulate Nedd4-2 interaction (e.g. ERK/MAPK and others (13)) may account for our observed binding results. Regardless of the underlying mechanism, it is clear that specific modulation of the Nedd4-2-β-ENaC interaction alone may be sufficient to alter ENaC currents in vivo, as a single β-ENaC point mutation that disrupts the PY motif causes Liddle’s syndrome (61). Thus, our binding results could explain the effect of AMPK on Na\(^+\) transport, but further characterization is warranted.

Cellular AMPK activation appeared to equally enhance the binding of Nedd4-2 to both mature and immature forms of β-ENaC under our experimental conditions (Fig. 10, center). This finding suggests that the AMPK effect does not depend on the degree of ENaC processing. A potential implication of these results is that AMPK, and therefore Nedd4-2, can regulate retrieval of ENaC regardless of the maturation state of the subunits. However, independent of AMPK status, there was a selective enrichment in the binding of cleaved or mature forms of all three ENaC subunits to Nedd4-2 in our studies (Fig. 9), suggesting that Nedd4-2 preferentially associates with a processed pool of ENaC in cells. Further mechanistic studies to investigate how and where Nedd4-2 interacts with ENaC in cells are warranted to expand on these findings.

Future work will focus on delineating the mechanisms of how AMPK enhances the interaction between Nedd4-2 and β-ENaC. Identifying the site(s) of AMPK-dependent phosphorylation of Nedd4-2 should help to elucidate this mechanism. Additional experiments to characterize the amplitude and time course of this response in a physiologic cell system will also aid our understanding of how AMPK regulates Na\(^+\) transport.

In summary, we have identified the ubiquitin-protein ligase Nedd4-2 as a novel target of AMPK phosphorylation, and our findings have shed further light on the mechanism of AMPK-dependent regulation of ENaC. AMPK serves as a metabolic sensor that may couple cellular energy status to both energy-generating processes and energy-expending processes such as ATP-dependent ion transport (28). Under conditions of metabolic stress, inhibition of ENaC-mediated Na\(^+\) influx should help prevent the dissipation of ionic gradients across the plasma membrane when Na\(^+\), K\(^+\)-ATPase function may be compromised. Of note, Nedd4-2 has been reported to regulate several transport proteins in addition to ENaC, including voltage-gated channels, Cl\(^-\)/Cl\(^-\) channels, glutamate transporters, and Na\(^+\)-coupled glucose and phosphate cotransporters, (62–65). Therefore, regulation of Nedd4-2 by AMPK may serve as a general mechanism to couple membrane transport to cellular metabolic status via ubiquitination.

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AMP-activated Kinase Inhibits the Epithelial Na\(^+\) Channel through Functional Regulation of the Ubiquitin Ligase Nedd4-2

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