The epithelial Na⁺ channel (ENaC) regulates epithelial salt and water reabsorption, processes that require significant expenditure of cellular energy. To test whether the ubiquitous metabolic sensor AMP-activated kinase (AMPK) regulates ENaC, we examined the effects of AMPK activation on amiloride-sensitive currents in Xenopus oocytes and polarized mouse collecting duct mENaC subunit-expressing cells. Microinjection of oocytes expressing mouse ENaC (mENaC) with either active AMPK protein or an AMPK activator inhibited mENaC currents relative to controls as measured by two-electrode voltage-clamp studies. Similarly, pharmacological AMPK activation or overexpression of an activating AMPK mutant in mENaC-expressing cells inhibited amiloride-sensitive short-circuit currents. Expression of a degenerin mutant β-mENaC subunit (S518K) along with wild type α and γ increased the channel open probability (Pₒ) to ~1. However, AMPK activation inhibited currents similarly with expression of either degenerin mutant or wild type mENaC. Single channel recordings under these conditions demonstrated that neither Pₒ nor channel conductance was affected by AMPK activation. Moreover, expression of a Liddle’s syndrome-type β-mENaC mutant (Y618A) greatly enhanced ENaC whole cell currents relative to wild type ENaC controls and prevented AMPK-dependent inhibition. These findings indicate that AMPK-dependent ENaC inhibition is mediated through a decrease in the number of active channels at the plasma membrane (N), presumably through enhanced Nedd4-2-dependent ENaC endocytosis. The AMPK-ENaC interaction appears to be indirect; AMPK did not bind ENaC in cells, as assessed by in vitro pull-down assays, nor did it phosphorylate ENaC in vitro. In summary, these results suggest a novel mechanism for coupling ENaC activity and renal Na⁺ handling to cellular metabolic status through AMPK, which may help prevent cellular Na⁺ loading under hypoxic or ischemic conditions.

The maintenance of transmembrane ion and solute gradients is a crucial process that permits normal cellular functioning, viability, and coordinated transepithelial transport but also consumes a substantial portion of total cellular energy (1). Under conditions of metabolic stress, the expression and activity of many membrane transport proteins including ENaC (2) are inhibited, thereby limiting the dissipation of ionic gradients and preserving the cellular energy required to maintain them (2). However, the cellular mechanisms that link membrane transport to energy production and metabolic status have remained elusive.

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 metabolic stress, in response to elevated intracellular AMP:ATP ratios (3). A parallel activation pathway involves phosphorylation of the α subunit by a recently identified upstream LKB1 kinase complex at a Thr residue in its activation loop (4). The earliest discovered substrates of AMPK were important metabolic enzymes, where it was found that AMPK acts to shut off ATP-utilizing anabolic pathways and turn on ATP-generating catabolic pathways under conditions of metabolic stress (3, 5). In addition, there has been a recent surge in reports linking AMPK to many other general cellular functions, including the control of apoptosis, cell growth and division, gene transcription, and protein synthesis (5). Modulation of AMPK may also play an important role in the pathogenesis and treatment of common disorders. For instance, the anti-diabetic drugs metformin and rosiglitazone act through AMPK activation via different cellular mechanisms (6, 7). Also, AMPK may play an important role in obesity, because leptin receptor signaling to enhance fatty acid breakdown in skeletal muscle and stimulation of appetite in the hypothalamus both require AMPK modulation (8, 9).

A novel function for AMPK that arose from our recent work is that of an ion transport regulator. We have recently described that AMPK interacts with the cystic fibrosis transmembrane conductance regulator (CFTR) in vivo and inhibits CFTR Cl⁻ channel activity in both Xenopus oocytes and polarized epithelial cells exposed to amiloride. These results are consistent with the notion that AMPK may be a sensor of metabolic status that regulates cellular Na⁺ handling via modulation of ENaC activity.

1 The abbreviations used are: ENaC, epithelial Na⁺ channel; AMPK, AMP-activated protein kinase; mENaC, mouse ENaC; CFTR, cystic fibrosis transmembrane conductance regulator; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5’-monophosphate; TEV, two-electrode voltage clamp; HA, hemagglutinin; GST, glutathione S-transferase; DEG, degenerin mutant; cRNA, complementary RNA; K-ZMP, potassium-ZMP; K-glucuronate, potassium-glucuronate; PBS, phosphate-buffered saline; ANOVA, analysis of variance; ERK, extracellular signal-regulated kinase; WT, wild type.
ampk-dependent inhibition of enac

epithelial cells, primarily through an inhibition of the cftr single-channel open probability (10–12). Because cftr requires atp for gating and is regulated by its phosphorylation state (13), the ampk-cftr interaction appears to provide a sensitive mechanism for the coupling of ion transport to cellular metabolic status.

enac is expressed in the apical plasma membrane of many epithelial tissues, including principal cells in the distal nephron of the kidney and epithelial cells in the urinary bladder, lung, distal colon, taste buds, and ducts of salivary and sweat glands (14). Working in conjunction with a basolateral na+, k+-atpase, these channels mediate the first step of active na+ reabsorption across epithelial cell layers and play a major role in the control of total body salt and water homeostasis, blood pressure, and airway surface liquid clearance. The physiological importance of enac is illustrated by naturally occurring mutations that cause increased or decreased enac activity, e.g., liddle’s syndrome, caused by a gain-of-function mutation and characterized by increased enac activity, and liddle’s syndrome, caused by a gain-of-function mutation and characterized by volume depletion, hypotension, and hyperkalemia (15). Moreover, gain of enac function caused by a loss of its normal regulation by cftr in the airways of patients with cystic fibrosis may be a major contributing factor in the pathogenesis of cystic fibrosis airway disease (16, 17).

enac is composed of three structurally related subunits, α, β, and γ. Each subunit shares ~30–40% sequence identity with the others and has two presumed membrane spanning domains, a large extracellular loop, and intracellular n and c termini (18). enacs have been traditionally identified by their sensitivity to inhibition by submicromolar concentrations of the diuretic amiloride, their high selectivity for conductance of na+ and li+ over k+, and “goldman-type” current-voltage relationships (18). enac genes are regulated by the action of the volume regulatory hormones aldosterone, vasopressin, and atrial natriuretic peptide (19–21), as well as other hormones such as insulin and endothelin (22, 23). A number of diverse mechanisms including the regulation of channel synthesis, intracellular channel trafficking, membrane insertion and retrieval, and single-channel properties (e.g. p) are important in controlling enac activity (18, 24). In particular, the interaction between the “py” motifs on the c termini of β- and γ-enac and the ww domains of nedd4-2, an intracellular ubiquitin-protein ligase that promotes internalization and subsequent degradation of enac, is emerging as an important locus for enac activity regulation by various mediators and signaling pathways (24). it has also long been recognized that inhibition of cellular metabolism through depletion of metabolic substrates (25, 26) and hypoxia (27, 28) inhibits apical na+ channel activity in epithelia, but the underlying mechanisms involved are unclear.

in this study we have examined the potential role of ampk in regulating enac. we found that activation of ampk inhibits enac activity in both the xenopus oocyte expression system and in polarized mouse cortical collecting duct (mpkcccd1α) cells. as assessed in oocytes, ampk stimulation decreases enac surface expression, presumably through effects on nedd4-2-mediated endocytic retrieval. ampk-dependent regulation of enac may link enac activity to cellular metabolic status and be involved in the inhibition of enac that occurs with metabolic depletion.

experimental procedures

reagents and chemicals—all of the chemicals used were obtained from sigma unless otherwise noted. 5-aminomidaole-4-carboxamide-1-β-d-ribofuranosyl 5-monophosphate (zmp) were obtained from tokyo research chemicals, inc. (nyc, canada). purified recombinant active rat ampk holozyme (α1-t172d, β1, γ1, or α1-t172d, β1, γ1-r80q) and inactive ampk holozyme (α1-d157a, β1, γ1) were synthesized in escherichia coli using a tri-cistronic plasmid containing the three genes in tandem and purified as described (29, 30). adenoviral vectors engineered to express kinase-dead rat ampk-α1-k45r and the constitutive-activating human ampk-γ1-r70q mutant subunits were kindly supplied by l. witters and amplified and purified by the university of pittsburgh vector core reagent program.

oocyte expression—maintaining Xenopus laevis frogs, surgical extraction of ovaries, and collagenase treatment of oocytes were carried out as described (31). complementary rnas (cRNAs) for α, β, and γ mouse wild type and mutant enac subunits were synthesized using the t3 mMessage mMACHINE kit (ambion, austin, tx). ampk cRNAs were synthesized as described previously (10). the purity and quantity of cRNA were assessed by agarose gel electrophoresis. Single- to vi oocytes were injected with 1–2 ng/subunit of enac cRNAs (along with 5 ng/subunit of ampk cRNAs for some experiments) within 1 day of collagenase treatment. the oocytes were then maintained in modified barth’s saline supplemented with antibiotics at 18 °c for 1–2 days before experimentation.

two-electrode voltage clamp—two-electrode voltage clamp (TEV) measurements were performed using a Warner oocyte clamp formed on a WC-752C (warner instruments, hamden, CT). the data were acquired through Clampex 8.0 (axon instruments, union city, CA) using a digitizer 1322A interface at 1 kHz and stored electronically on a PC hard disk. Micropipettes had resistances of 0.3–3 MΩ when filled with 3 M KCl. the oocytes were maintained in a recording chamber (RC-32, Warner Instruments) and continuously perfused with TEV solution containing 110 mM NaCl, 2 mM KCl, 2 mM CaCl2, and 10 mM Hepes, pH 7.4 (adjusted with phosphoric acid), for oocyte injections (23 nl/oocyte). the concentration of purified active or inactive AMPK holozyme was 5 μg/μl. activity of the purified kinase was verified after dialysis using an in vitro kinase assay, as described previously (32).

cell-attached patch clamp measurements—1.5–4 h after microinjection, expressing oocytes with either K-glucuronate or K-Zmp and after removal of their vitelline membranes, single channel patch-clamp recordings in the cell-attached mode were performed using identical bath and pipette solutions with LiCl as the predominant salt, as previously described (33). Cell-attached recordings were generally performed at +60 mV (pipette potential) except when gathering data for I-V plots to determine single-channel conductance.

Cell Culture and Adenoviral Transduction—HEK-293 cells were maintained in dulbecco’s modified eagle’s medium (BioWhittaker) with 4.5 g/liter glucose and l-glutamine, 10% fetal bovine serum, and penicillin-streptomycin. Cells were seeded at confluent density into 6-well plates 1 day prior to transfection for phosphorylation and GST pull-down experiments. Mouse cortical collecting duct (mpkcccd1α) cells obtained from alain vandewalle (paris, france) were cultured in defined media containing fetal bovine serum, antibiotics, and other hormones and nutrients as previously described (34). the cells were seeded at confluent density onto 1.2-cm diameter Transwells (costar, corning, ny) and grown for at least 3 days prior to adenoviral transduction. purified adenoviral vector diluted in Ca2+-free PBS to a multiplicity of infection of 300 in a volume of 1 μl was added to the apical compartment after washing and incubating both the apical and basolateral compartments in Ca2+-free PBS for 30 min at 37 °C. After 90 min of further incubation with virus at 37 °C, the solutions were washed and replaced with fresh medium. Mock transductions using Ca2+-free PBS were also performed in parallel. the experiments were performed 2 days after adenoviral transduction.

Equivalent Short Circuit Current Measurements—A portable epithelial
volts ohmmeter (World Precision Instruments, Sarasota, FL) was used to measure equivalent short circuit currents (Isc), as described (35). Amiloride (20 \( \mu M \)) was added at the end of each experiment to derive the amiloride-sensitive component of the calculated short circuit current. Typically, \( \geq 90\% \) of the total Isc was inhibited after amiloride addition.

**Glutathione S-transferase Pull-down Assays**—HEK-293 cells were co-transfected with a plasmid expressing either: V5-epitope tagged \( \alpha\), \( \beta\), or \( \gamma\)-ENaC (36) or hemagglutinin (HA)-tagged AMPK-\( \beta1\) subunit (pMT2-AMPK-\( \beta1\)) along with a plasmid expressing either glutathione S-transferase (GST) alone (pEBC) or N-terminal GST-tagged AMPK-\( \alpha1\) subunit (pEG-B-AMPK-\( \alpha1\)) (37). Subsequent in vivo binding studies were performed essentially as described previously (10), except that sample lysates were maintained at room temperature until affinity purification with glutathione-Sepharose beads (Pierce). ENaC samples were probed with a primary monoclonal anti-V5 antibody (1:5000; Invitrogen), and AMPK-\( \beta1\) samples were probed with a primary monoclonal anti-HA antibody (1:1000; Covance). A secondary horseradish peroxidase-conjugated goat anti-mouse antibody (1:5000; Amersham Biosciences) was then used for both. All of the membranes were then reprobed with anti-GST-horseradish peroxidase antibody to visualize and quantitate GST fusion protein expression in all lanes. The intensity of all relevant bands was quantitated using a Bio-Rad Versadoc Imager. The percentage of GST pull-down for each condition was calculated from densitometric band intensities in the bound lanes divided by the corresponding band intensity in the total lysate lanes, corrected for the percentage of lysate loaded (5%). The binding of GST-AMPK-\( \alpha1\) to ENaC relative to that of GST alone (background nonspecific binding) was determined by comparing the percentage of GST pull-down under the two conditions normalized to the relative GST fusion protein expression in the bound lanes under the two conditions.

**In vitro Phosphorylation Assays**—HEK-293T cells were transfected with V5-tagged \( \alpha\), \( \beta\), or \( \gamma\)-tagged mENaC subunits (36) or wild type CFTR and lysed 1 or 2 days after transfection, respectively. ENaC subunits were immunoprecipitated from lysates using the anti-V5 antibody or the m24–1 anti-CFTR monoclonal antibody coupled to protein A/G beads (Pierce), as described (10, 36). In vitro phosphorylation was performed using 1 \( \mu g/J\) purified active AMPK holoenzyme \( (\alpha\beta\gamma)\) or inactive AMPK holoenzyme \( (\alpha1\beta1\gamma1)\) previously synthesized in E. coli (30) with \( [\gamma-32P]ATP\) labeling as previously described for CFTR phosphorylation in Chinese hamster ovary cells (10). As a positive control for ENaC phosphorylation, parallel in vitro phosphorylations were performed using purified carboxy terminus kinase-2 (Promega), as per the manufacturer's recommendations. After SDS-PAGE and transfer to nitrocellulose, the membranes were first immunoblotted for expression of ENaC or CFTR. Then, after the enhanced chemiluminescent signal had decayed, phosphorylated bands were imaged by autoradiography. 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 per condition. Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls post hoc test. Statistical analyses were performed using ANOVA followed by Student-Newman-Keuls post hoc test. In all cases, \( p\) values < 0.05 were considered significant.

**RESULTS**

**AMPK Activation Inhibits ENaC Currents Expressed in Oocytes**—We have recently shown that the metabolic-sensing kinase AMPK inhibits the CFTR Cl\(^{-}\) channel by inhibiting channel gating, providing a new potential mechanism for the coupling of ion transport to cellular metabolic status (10–12). ENaC at the apical membrane, in concert with the basolateral Na\(^{+}\)/K\(^{-}\)-ATPase, plays a critical role in salt and fluid reabsorption in the kidney and lung, processes that occur at high cellular metabolic expense (2, 38). It is thus of interest to determine whether AMPK may also regulate ENaC channel activity. We initially utilized the *Xenopus* oocyte expression system to measure changes in amiloride-sensitive ENaC currents and ENaC surface expression as a function of AMPK activation.

AMPK-dependent changes in the whole cell currents of oocytes expressing mENaC were measured using the TEV method. Typical current-time sweeps recorded with voltage steps from -140 to +60 mV are shown 1–3 h after microinjection of either purified catalytically inactive or highly active AMPK holoenzyme mutants (Fig. 1, A and B). These whole cell currents measured in mENaC-expressing oocytes were almost completely sensitive to 10 \( \mu M\) amiloride (lower sweeps), suggesting only a minimal contribution of other ion transport pathways to the whole cell currents measured in oocytes under these conditions. I-V plots obtained from current readings in the traces shown in Fig. 1 (A and B) demonstrated the expected linear relationship for ENaC conductance (Fig. 1C). The difference in currents measured with transmembrane voltage clamped at \(-100\) mV before and after amiloride treatment (amiloride-sensitive ENaC currents) was calculated for each experiment and compared under the two conditions. Relative amiloride-sensitive currents measured from multiple experiments are shown in Fig. 1D. The injection of active AMPK inhibited the ENaC currents by \( \sim 45\%\) relative to inactive AMPK-injected controls, suggesting that AMPK inhibits ENaC activity in oocytes expressing mENaC.

As an independent method to acutely activate AMPK in the oocytes, the AMP analogue ZMP, which is the active intracellular metabolite of the AMPK activator AICAR (39), or control solution was microinjected into oocytes, and amiloride-sensitive ENaC currents were measured 1–3 h after injection (Fig. 2). In oocytes expressing wild type AMPK, K-ZMP inhibited ENaC currents by \( \sim 40–55\%\), as compared with K-gluconate (Fig. 2A) or water (Fig. 2B) microinjection as controls. The degree of ZMP-mediated inhibition was dose-dependent (not shown) and time-dependent (Fig. 2C), with growing inhibition over a 20-h post-injection observation period. Similar inhibition was also observed without exogenous AMPK cRNA expression in the oocytes (Figs. 3 and 5), suggesting that endogenous *Xenopus* AMPK is present in the oocytes and is activated by ZMP. However, this ZMP-mediated inhibition of ENaC was not observed in oocytes overexpressing the kinase-dead, dominant-negative AMPK-\( \alpha1\)-K45R mutant subunit (Fig. 2D), suggesting that the ZMP effect was mediated specifically through AMPK activation. Because the *X. laevis* AMPK catalytic subunit is highly homologous (with 90% identical and 96% conserved residues) to the rat isofrom used in these *Xenopus* oocyte expression studies,\(^*\) we expected that overexpression of exogenous rat \( \alpha1\)-K45R subunit would bind endogenous \( \beta\) and \( \gamma\) and displace endogenous *a Xenopus* subunits from the holoenzyme complex, thereby having a dominant-negative effect. This mechanism of dominant-negative AMPK inhibition through overexpression of the kinase-dead mutant \( \alpha\) subunit has been described previously (40).

**AMPK-dependent ENaC Inhibition Is Not Mediated via Effects on ENaC Single-channel Properties in Oocytes**—To test whether AMPK-dependent inhibition of ENaC occurs through effects on the number of active ENaCs expressed at the plasma membrane \( (N)\), we initially performed antibody surface labeling experiments on oocytes expressing extracellular epitope-tagged \( \beta\)-ENaC, as described previously (41). However, the injection of oocytes with ZMP or control solution within a few hours of starring the labeling procedure caused a high level of nonspecific oocyte labeling, rendering comparisons unreliable (data not shown). As an alternate approach to determine the mechanism of AMPK-dependent ENaC inhibition, we compared TEV currents in oocytes expressing wild type mENaC with oocytes expressing a DEG mutant \( \beta\)-mENaC subunit (\( \beta\)-S518K) along with wild type \( \alpha\) and \( \gamma\)-mENaC (Fig. 3). This DEG gating mutant is homologous to the human \( \beta\)-S520K mutant used by Snyder et al. (42) and has been previously shown to increase the \( P_{o}\) of ENaC to nearly 1 (43). As expected, the number of oocytes measured, and \( N\) indicates the number of oocyte batches used. StatView (SAS Institute Inc., Cary, NC) was used for statistical analyses. In all cases, \( p\) values < 0.05 were considered significant.

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\(^*\) Analysis was performed using an Entrez protein data base search (www.ncbi.nlm.nih.gov).
AMPK-dependent Inhibition of ENaC

oocytes expressing the DEG mutant β subunit had significantly enhanced amiloride-sensitive ENaC currents to over twice that of controls. However, injection of ZMP to activate AMPK inhibited ENaC currents by ~50% compared with inactive AMPK-injected controls (*, p < 0.001, ANOVA; n = 35–42, N = 4). The data shown are the mean (± S.E.) amiloride-sensitive currents normalized to the mean amiloride-sensitive current of inactive AMPK-injected oocytes for each experiment.

To rule out the possibility that ZMP treatment was unexpectedly affecting the gating or conductance properties of this mutant channel, single-channel patch-clamp recordings were performed in the cell-attached mode using oocytes expressing the DEG mutant channel after injection with either K-gluconate or K-ZMP (Fig. 4). Parallel TEV measurements were performed on oocytes from the same batch to confirm that there was inhibition of ENaC whole cell currents by ZMP, as shown in Fig. 3. Typical traces and amplitude histograms from patches performed on oocytes injected with either K-gluconate (Fig. 4A) or K-ZMP (Fig. 4B) demonstrate that the channels were largely locked in the open state with only occasional brief channel closures under both conditions (thereby having a P_o of nearly 1). These traces appear very similar to those shown previously for this mutant (43). Apparent ENaC single-channel conductance was also unaffected by ZMP treatment, because the slopes of I-V fits under the two conditions were very similar (Fig. 4C). Taken together, the results shown in Figs. 3 and 4 suggest that AMPK-dependent ENaC inhibition is mediated through effects on surface expression of active ENaC channels at the plasma membrane.

AMPK May Enhance Endocytic Retrieval of ENaC from the Plasma Membrane—As another approach to test the effect of AMPK on ENaC surface expression and trafficking in oocytes, we expressed a β-Y618A Liddle’s syndrome-type mutant mENaC subunit along with wild type α- and γ-mENaC and compared whole cell currents in these oocytes relative to currents in oocytes expressing wild type mENaC channels as a function of AMPK stimulation. This point mutation in the β subunit eliminates the consensus PY motif that is required for Nedd4-2 interaction with ENaC and has been shown to enhance ENaC surface expression by disrupting Nedd4-2-dependent ENaC endocytosis and degradation (44, 45). As expected, expression of this mutant dramatically enhanced ENaC whole cell currents relative to wild type ENaC injected controls by a factor of ~3 (Fig. 5). Interestingly, AMPK activation through ZMP injection failed to inhibit ENaC-dependent whole cell currents in oocytes expressing the β-Y618A mutant subunits, whereas this inhibition was still observed with wild type ENaC (Fig. 5). This result suggests that ENaC inhibition of AMPK is mediated via a decrease in N, which may occur through enhancement of Nedd4-2-dependent endocytic retrieval of ENaC from the plasma membrane.

AMPK Inhibits Endogenous ENaC Activity in Polarized Collecting Duct Cells—The Xenopus oocyte is a convenient system to use for the controlled expression of transport proteins such as ENaC and their cofactors and subsequent electrophysiological measurements. However, because the oocyte is a heterologous expression system, it is desirable to confirm the effects
within each experiment. Microinjection of 23–32 nl of 40 mM K-ZMP versus K-glucocinate (A) or 40 mM K-ZMP versus distilled water (B) into oocytes expressing mENaC and wild type α1, β1, and γ1 rat AMPK subunits was performed. Amiloride-sensitive currents measured 1–3 h after injection were significantly inhibited in K-ZMP-injected oocytes compared with controls (*, p < 0.001, ANOVA, n = 24–31). C, time course of ZMP-dependent inhibition of amiloride-sensitive currents. TEV measurements were performed prior to (time 0) and at various times points after injection with 23 nl of either 40 mM K-ZMP or K-glucocinate. The data points shown represent the means (±S.E.) of TEV measurements on 8–10 oocytes at each time point (*, p < 0.01 compared with K-glucocinate-injected oocytes at same time point, unpaired t tests). D, K-ZMP-mediated inhibition was not observed in oocytes expressing mENaC + kinase-dead (α1-K45R, β1, γ1) rat AMPK (p = 0.85; n = 11–17, N = 2). For A, B, and D, the data shown are the mean (±S.E.) amiloride-sensitive currents normalized to the mean amiloride-sensitive current measured for control-injected oocytes within each experiment.

FIG. 2. Effects of injection of pharmacologic AMPK activator on ENaC currents in oocytes. Microinjection of 23–32 nl of 40 mM K-ZMP versus K-glucocinate (A) or 40 mM K-ZMP versus distilled water (B) into oocytes expressing mENaC and wild type α1, β1, and γ1 rat AMPK subunits was performed. Amiloride-sensitive currents measured 1–3 h after injection were significantly inhibited in K-ZMP-injected oocytes compared with controls (*, p < 0.001, ANOVA, n = 24–31, N = 4; #, p < 0.05, ANOVA, n = 15, N = 3). C, time course of ZMP-dependent inhibition of amiloride-sensitive currents. TEV measurements were performed prior to (time 0) and at various times points after injection with 23 nl of either 40 mM K-ZMP or K-glucocinate. The data points shown represent the means (±S.E.) of TEV measurements on 8–10 oocytes at each time point (*, p < 0.01 compared with K-glucocinate-injected oocytes at same time point, unpaired t tests). D, K-ZMP-mediated inhibition was not observed in oocytes expressing mENaC + kinase-dead (α1-K45R, β1, γ1) rat AMPK (p = 0.85; n = 11–17, N = 2). For A, B, and D, the data shown are the mean (±S.E.) amiloride-sensitive currents normalized to the mean amiloride-sensitive current measured for control-injected oocytes within each experiment.

AMPK Does Not Bind or Phosphorylate ENaC Subunits—AMPK-dependent regulation of ENaC is physiologically relevant in polarized epithelial cells.

have been used as a model epithelial system for ENaC study in vitro (34).

We have used three independent methods to activate AMPK in polarized mpkCCD14 cells and tested the effects on amiloride-sensitive equivalent Isc (Fig. 6). Treatment for 24 h with either 1 mM AICAR (Fig. 6A) or 2 mM metformin (Fig. 6B), each activating endogenous AMPK through different mechanisms (7), resulted in a significant 40–45% inhibition in amiloride-sensitive Isc. We also transduced these polarized monolayers with purified adenoviral constructs to express mutant AMPK subunits, either the catalytically inactive dominant-negative α1-K45R subunit or the constitutive-activating γ1-R70Q subunit (32). Amiloride-sensitive Isc was measured 2 days after transduction. Activation of endogenous AMPK by overexpression of the γ1-R70Q AMPK subunit inhibited ENaC Isc by 37% as compared with mock-infected and 31% as compared with α1-K45R-infected monolayers (Fig. 6C). Control observations using a green fluorescent protein-adenovirus construct to infect these cells at the same multiplicity of infection revealed that only ~50% of the cells expressed detectable levels of exogenous protein (not shown), which may partially account for the reduced inhibition with adenoviral expression as compared with the use of AICAR or metformin. Of note, all of the experiments shown in Fig. 6 support the conclusion that activation of endogenous AMPK in polarized kidney collecting duct cells inhibits endogenous ENaC activity and suggest that the AMPK-dependent regulation of ENaC is physiologically relevant in polarized epithelial cells.
direct regulation, we have asked whether AMPK binds to or directly phosphorylates ENaC. As previously used to demonstrate AMPK binding to CFTR in CFTR-expressing cells (10), in vivo GST pull-down assays were performed to determine whether V5 epitope-tagged \(\alpha\)-, \(\beta\)-, or \(\gamma\)-ENaC subunits bind to GST-tagged AMPK-\(\alpha\) expressed in HEK-293 cells (Fig. 7). None of the ENaC subunits bound to GST-tagged AMPK-\(\alpha\) expressed in cells with any greater affinity than background binding to GST alone, and this nonspecific binding constituted \(\sim\)1% of all ENaC present in the cell lysates (Fig. 7A and Table I). Serving as a positive control for this assay, we found that HA-tagged AMPK-\(\beta\) did bind with high specificity to GST-tagged AMPK-\(\alpha\) (11.58 ± 1.24% of total cellular AMPK-\(\beta\) bound versus 0.18 ± 0.03% for GST alone) (Fig. 7B and Table I). These results demonstrate that none of the ENaC subunits appear to bind, either directly or indirectly, to the catalytic AMPK subunit in intact cells. Preliminary co-immunoprecipitation experiments performed on lysates from cells expressing HA-tagged AMPK-\(\beta\) or \(\gamma\) subunits along with V5-tagged ENaC subunits similarly failed to demonstrate any specific interaction between these AMPK regulatory subunits and ENaC (not shown).

Based on the reported preferred consensus motif for AMPK phosphorylation (5), there are no conforming AMPK phosphorylation sites on the presumed cytoplasmic domains of any of the three mENaC subunits. To confirm that ENaC is not a target for AMPK-dependent phosphorylation in vitro, V5...
epitope-tagged ENaC subunits transiently expressed in HEK-293 cells were immunoprecipitated from lysates, and in vitro phosphorylation experiments were performed using recombinant purified active (α1-T172D, β1, γ1) or kinase-dead (α1-D157A, β1, γ1) AMPK holoenzyme (Fig. 8). Under these conditions there was no enhancement in 32P incorporation into β-ENaC with active AMPK, suggesting that AMPK does not phosphorylate β-ENaC in vitro (Fig. 8A, right panel). Casein kinase 2, which is known to phosphorylate β- and γ-ENaC (46), served as a positive control for ENaC phosphorylation in this assay (Fig. 8A). As a positive control for AMPK activity, immunoprecipitated CFTR was phosphorylated by active AMPK (Fig. 8A, left panel), consistent with our previous results (10). The same membrane immunoblotted for CFTR and the V5 epitope tag on β-ENaC demonstrated equivalent expression of CFTR or ENaC across conditions (Fig. 8B). Similar experiments have also failed to demonstrate AMPK-dependent phosphorylation of α- and γ-ENaC (not shown). Thus, it appears that the AMPK-dependent inhibition of ENaC does not occur through direct interaction with or phosphorylation of ENaC.

**DISCUSSION**

In this study we found that ENaC activity is regulated by the metabolic-sensing kinase AMPK in both the heterologous Xenopus oocyte expression system and in polarized mouse CCD cells that endogenously express both proteins. As shown by parallel whole cell TEV and single-channel patch-clamp studies utilizing the β-S518K DEG ENaC mutant (Figs. 3 and 4), changes in single channel properties (P", and conductance) cannot account for the AMPK-dependent inhibition of ENaC, suggesting that the inhibition is mediated by changes in N", the expression of active channels at the plasma membrane. The lack of AMPK-dependent inhibition of the Liddle’s syndrome type mutant ENaC subunit (Fig. 5) supports this conclusion and further suggests that AMPK-mediated inhibition of ENaC plasma membrane expression occurs through enhancement of Nedd4-2-dependent endocytic retrieval. The lack of apparent AMPK-ENaC interaction in cells (Fig. 7) or AMPK-dependent phosphorylation in vitro (Fig. 8) suggests that the mechanism for inhibition is indirect and thus potentially mediated through other cellular signaling pathways that regulate ENaC.

Because the AMPK-dependent regulation of ENaC appears to be indirect, it is of interest to consider which intermediate proteins or pathways might transduce the inhibitory signal from AMPK to ENaC. Our data suggest that the Nedd4-2 endocytic retrieval pathway may be activated by AMPK, and recent studies implicate the importance of several signaling pathways in the regulation of Nedd4-2-dependent ENaC retrieval. AMPK could potentially target Nedd4-2 either directly or indirectly through effects on upstream Nedd4-2 regulators such as the serum and glucocorticoid-regulated kinase, which inhibits ENaC-Nedd4-2 interaction and thus promotes ENaC plasma membrane expression (45). Serum and glucocorticoid-regulated kinase is activated transcriptionally by aldosterone and through the insulin-dependent phosphoinositide 3-kinase pathway (47). Because AMPK is known to modulate the transcription of certain genes and protein synthesis and may be involved in cross-talk with the phosphoinositide 3-kinase pathway (5, 48), serum and glucocorticoid-regulated kinase activity could conceivably be regulated by AMPK through one or more of these mechanisms.

Phosphorylation of the C-terminal cytoplasmic tail of ENaC in the vicinity of the PY motif by kinases such as ERK and GRK2 is another general mechanism that regulates Nedd4-2-dependent ENaC endocytosis and degradation, presumably by modulating the binding affinity of the ENaC tail for Nedd4-2 (49, 50). Protein kinase C activation has been shown to inhibit ENaC, both by effects on ENaC gating (51) and by effects on endocytic retrieval, which may be mediated through the ERK pathway (52). From the standpoint of AMPK and relevant to ENaC regulation, atypical protein kinase Cs, the proline-rich tyrosine kinase-2, the ERK and p38 MAPK pathways, and phospholipase D have all been implicated as downstream effectors involved in AMPK-stimulated glucose uptake in skeletal muscle and AMPK effects in other systems (53–55). Thus, there are numerous possible mechanisms by which ENaC regulation may be linked to AMPK. Investigation of the intermediate pathways and mechanisms involved are important goals for further study.

ENaC in the distal nephron is a finely tuned regulator of Na+ reabsorption and thus total body salt and volume homeostasis. However, a prerequisite at the cellular level for active salt reabsorption is sufficient metabolic energy to drive the pumps that maintain the normal transmembrane electrochemical gradients required for transepithelial transport and other critical cellular processes. In actively transporting epithelial tissues, ion transport accounts for a large percentage of total cellular energy expenditure. For example, it has been estimated that the Na+, K+-ATPase alone utilizes up to 50% of total mitochondrial oxidative metabolism in renal tubular cells under resting conditions (38). If cellular “leak” pathways (e.g., ion channels and other downhill
and then immunoprecipitated from cell lysates for AMPK or casein kinase 2 (CK-2). The samples representing 5% of the total cellular lysate (lanes L) were loaded alongside samples eluted from the GSH-Sepharose beads (lanes B). The blots shown are representative of four replicate experiments. Quantitation of the binding under each condition is shown in Table I.

\begin{table}[h]
\centering
\caption{Percentage of binding of various subunits corrected for GST Fusion protein expression}
\begin{tabular}{|c|c|c|c|c|}
\hline
& $\alpha$-ENaC & $\beta$-ENaC & $\gamma$-ENaC & AMPK-\beta1 \\
\hline
GST alone & 0.85 ± 0.17 & 0.37 ± 0.10 & 0.22 ± 0.09 & 0.18 ± 0.03 \\
GST-AMPK-\alpha1 & 0.41 ± 0.13 & 0.64 ± 0.24 & 0.41 ± 0.21 & 11.58 ± 1.24 \\
\hline
\end{tabular}
\end{table}

Our finding that AMPK regulates ENaC provides a new potential mechanism for the coupling of ENaC activity to cellular metabolic state. Specifically, ENaC inhibition by AMPK may help prevent excessive Na\(^{+}\) influx under conditions of metabolic stress, limiting the need for ongoing active pumping of Na\(^{+}\) out of cells by the Na\(^{+}\),K\(^{-}\)-ATPase. It has long been recognized that hypoxia and metabolic uncoupling (or ischemia) down-regulate apical Na\(^{+}\) channel activity in epithelia (25–28), although the underlying mechanisms for this regulation have been unclear. Further studies are warranted to more directly test the potential role of AMPK in the inhibition of ENaC that occurs following ischemia and hypoxia. It is important to note that AMPK may have tonic activity under normal conditions, and its activity is exquisitely sensitive to even minor fluctuations in cellular nucleotide levels (56). Thus, AMPK may play an important role in regulating ion transport not only under pathological conditions like ischemia or hypoxia but also in responding to normal physiological fluctuations in cellular ATP levels. Finally, because epithelial channels such as ENaC and CFTR play a key role in modulating salt and water reabsorption and secretion across epithelial tissues in the kidney, lung, and other organs, AMPK may play a general role in linking these processes to underlying cellular metabolic status.

In summary, AMPK has a growing and diverse list of cellular targets, including membrane transport proteins such as glucose transporters (57, 58), CFTR (10), voltage-gated Na\(^{+}\) channels (59), and now ENaC. AMPK-dependent inhibition of plasma membrane “leak” pathways like CFTR and ENaC may play an adaptive role in limiting cellular energy expenditure under conditions of metabolic depletion. Concomitantly, AMPK-dependent stimulation of plasma membrane glucose and fatty acid uptake pathways help provide more cellular fuel to replete energy stores (60). Thus, as found for its actions on other cellular targets, the effects of AMPK on membrane transport proteins appear consonant with its role as a homeostatic regulator of cellular energy balance.

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Epithelial Sodium Channel Inhibition by AMP-activated Protein Kinase in Oocytes and Polarized Renal Epithelial Cells

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