Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells

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Hallows, Kenneth R., Gary P. Kobinger, James M. Wilson, Lee A. Witters, and J. Kevin Foskett. Physiological modulation of CFTR activity by AMP-activated protein kinase in polarized T84 cells. Am J Physiol Cell Physiol 284: C1297–C1308, 2003. First published January 2, 2003; 10.1152/ajpcell.00227.2002.—The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated, ATP-gated Cl− channel and cellular conductance regulator, but the detailed mechanisms of CFTR regulation and its regulation of other transport proteins remain obscure. We previously identified the metabolic sensor AMP-activated protein kinase (AMPK) as a novel protein interacting with CFTR and found that AMPK phosphorylated CFTR and inhibited CFTR-dependent whole cell conductances when coexpressed with CFTR in Xenopus oocytes. To address the physiological relevance of the CFTR-AMPK interaction, we have now studied polarized epithelia and have evaluated the localization of endogenous AMPK and CFTR and measured CFTR activity with modulation of AMPK activity. By immunofluorescent imaging, AMPK and CFTR share an overlapping apical distribution in several rat epithelial tissues, including nasopharynx, submandibular gland, pancreas, and ileum. CFTR-dependent short-circuit currents (Isc) were measured in polarized T84 cells grown on permeable supports, and several independent methods were used to modulate endogenous AMPK activity. Activation of endogenous AMPK with the cell-permeant adenosine analog 5-amino-4-imidazolecarboxamid-1-β-D-ribofuranoside (AICAR) inhibited forskolin-stimulated CFTR-dependent Isc in nonpermeabilized monolayers and monolayers with nystatin permeabilization of the basolateral membrane. Raising intracellular AMP concentration in monolayers with basolateral membranes permeabilized with α-toxin also inhibited CFTR, an effect that was unrelated to adenosine receptors. Finally, overexpression of a kinase-dead mutant AMPK-α1 subunit (α1-K45R) enhanced forskolin-stimulated Isc in polarized T84 monolayers, consistent with a dominant-negative reduction in the inhibition of CFTR by endogenous AMPK. These results indicate that AMPK plays a physiological role in modulating CFTR activity in polarized epithelia and suggest a novel paradigm for the coupling of ion transport to cellular metabolism.

THE CYSTIC FIBROSIS (CF) transmembrane conductance regulator (CFTR) is localized to the apical membrane of epithelial cells lining various tissues, including the lungs, gastrointestinal tract, exocrine pancreas, and sweat ducts (62). CF is associated with abnormal epithelial solute and fluid transport due to mutations in CFTR that reduce its plasma membrane expression or activity (58, 61). CFTR is a cAMP-dependent protein kinase (PKA)-activated, ATP-gated Cl− channel that belongs to the ATP-binding cassette (ABC) family of transporters. A unique feature of CFTR is the requirement for ATP binding and hydrolysis to support channel activity. It has been proposed that this ATP requirement may enable CFTR activity to be coupled to cellular metabolism (21, 47). In addition to its role as an apical membrane Cl− channel, it appears that CFTR also acts as a cellular “conductance regulator,” coordinating transepithelial solute and fluid transport by modulating the activities of several other plasma membrane transport proteins (35). The mechanisms involved in this modulation by CFTR are not yet clear but in some cases could involve CFTR-dependent ATP efflux from the cell (14, 50, 56). CFTR might also influence activities of other transport pathways by direct or indirect protein-protein interactions (35).

Several recently identified CFTR-interacting proteins may be important in regulating CFTR channel activity and/or plasma membrane expression, including syntaxin 1A, via its interaction with the NH2-terminal tail of CFTR (8, 45, 46), the PDZ domain-containing proteins NHERF (43, 48, 52) and CAP70 (59), and the μ2-subunit of the AP-2 adaptor protein complex (60), via interactions with the COOH-terminal tail. We discovered (26) that the α1 (catalytic)-subunit of the AMP-activated protein kinase (AMPK) was a strong and consistent interactor with the COOH tail of CFTR in a yeast two-hybrid screen.

AMPK is a serine/threonine kinase that exists as a heterotrimer composed of a catalytic α-subunit and regulatory β- and γ-subunits. Multiple isoforms (α1, α2, β1, β2, γ1, γ2, and γ3) exist, with differing tissue...
distributions and presumed substrates (33) and orthologs in all eukaryotes (28). The \( \alpha \)-subunit contains an \( \text{NH}_2 \)-terminal catalytic domain and a \text{COOH}-terminal regulatory domain involved in interactions with the \( \beta \)- and \( \gamma \)-subunits. The kinase activity increases during conditions of metabolic stress, in response to elevated intracellular AMP-to-ATP ratios (30). Activation of the kinase involves both the binding of AMP to allosteric site(s), probably involving both the \( \alpha \)- and \( \gamma \)-subunits (7, 63), and phosphorylation by an upstream AMPK kinase (29). Kinase activity is also regulated by association of the \( \beta \)- and \( \gamma \)-subunits, binding of which relieves an autoinhibitory interaction between the catalytic and regulatory domains of the \( \alpha \)-subunit (12). The earliest discovered substrates of AMPK were important rate-limiting biosynthetic enzymes (e.g., HMG-CoA reductase and acetyl-CoA carboxylase; Ref. 28). Phosphorylation by AMPK inhibits their enzymatic activities, thereby acting to preserve cellular ATP stores under conditions of metabolic depletion. Consequently, AMPK is believed to act as a metabolic sensor in cells, responding to changes in cellular energy charge by regulating ATP-utilizing and -generating metabolic pathways (28). In addition, there has been a recent surge in reports linking AMPK to other cellular functions, e.g., the regulation of glucose-dependent gene expression in pancreatic \( \beta \)-cells, cellular glucose uptake in contracting muscle, and the activation of endothelial nitric oxide synthase during tissue hypoxia (9, 36, 37). Furthermore, mutations in AMPK have been linked to diseases such as familial hypertrophic cardiomyopathy (5) and the Wolff-Parkinson-White syndrome (23). Finally, modulation of AMPK may play an important role in the pathogenesis and treatment of common disorders such as type II diabetes mellitus (64) and obesity (40).

CFTR is linked to cellular ATP in several important ways. CFTR consumes ATP in its gating, is regulated by its phosphorylation state, and may modulate ATP efflux from cells (14). Thus we considered that the interaction between CFTR and the metabolic sensor AMPK could have potential significant cell physiological implications. Although we found (26) that the regulatory domain of the AMPK \( \alpha \)-subunit binds the CFTR \text{COOH}-terminal tail, and that AMPK phosphorylated CFTR in vitro and inhibited cAMP-activated CFTR conductances in Xenopus oocytes, the physiological relevance of this protein-protein interaction has not yet been demonstrated in epithelial tissues and cells that endogenously express both proteins and in which CFTR plays an important transport role. In this study we examined the localization of endogenous CFTR and AMPK in various epithelial tissue sections. In addition, we used several approaches to modulate endogenous AMPK activity and then measured the effects on CFTR-dependent short-circuit currents (\( I_{sc} \)) in polarized T84 cell monolayers. The T84 secretory epithelial cell line, derived from a human colon adenocarcinoma, has been extensively characterized with respect to CFTR activity and function (3). Our results suggest that AMPK inhibits CFTR \text{Cl}\textsuperscript{−} channel activity in polarized T84 cells. This AMPK-dependent inhibition of CFTR may be physiologically relevant in general for polarized epithelia, providing a novel mechanism for the modulation of CFTR activity as a function of cellular metabolic state.

**EXPERIMENTAL PROCEDURES**

**Reagents and chemicals.** All reagents and chemicals used were purchased from Sigma (St. Louis, MO) unless otherwise noted.

**Immunofluorescent staining.** The preparation of various rat tissues and immunofluorescent staining of frozen sections were performed with rabbit polyclonal primary anti-\( \alpha \)-AMPK antibodies (54) or anti-CFTR-COH-terminal tail antibodies (pAbC-term.B, a generous gift from Dr. John Marshall, Genzyme Pharmaceuticals, Cambridge, MA) and biotinylated secondary anti-rabbit IgG along with rhodamine-conjugated avidin (Boehringer-Mannheim, Indianapolis, IN) exactly as described previously (26). For each tissue shown, contiguous sections were used for immunostaining AMPK-\( \alpha \) and CFTR so that similar structures could be compared. Hematoxylin and eosin staining of additional frozen sections from the same block was also performed for reference to aid in the identification of tissue structures. The tissues were obtained from the Morphology Core of the Institute for Gene Therapy, University of Pennsylvania.

**Ussing chamber \( I_{sc} \) measurements.** T84 cells were seeded at confluent density on 1.0-cm permeable supports (Costar Snapwells no. 3407) and grown in DMEM-F-12 medium. All medium was removed and replaced with fresh medium every 24–48 h. T84 cell monolayers were grown with medium on both the apical and basolateral sides, and inserts were used for Ussing chamber experiments 5–12 days after seeding the cells. For experiments using intact T84 cell monolayers (see Figs. 2 and 5), the experimental bath solutions contained (in mM) \( 120 \) NaCl, \( 25 \) NaHCO\(_3\), \( 3.3 \) KH\(_2\)PO\(_4\), \( 0.8 \) K\(_2\)HPO\(_4\), \( 1.2 \) MgCl\(_2\), \( 1.2 \) CaCl\(_2\), and 10 glucose. Mannitol was substituted for glucose in the apical bath to eliminate the contribution of Na\textsuperscript{+}-glucose cotransport to \( I_{sc} \), as previously described (17). The pH of this solution was 7.4 when gassed with a mixture of 95% O\(_2\)-5% CO\(_2\) at 37°C. For each experiment two paired Snapwell inserts were mounted in Ussing chambers interfaced with a voltage-current clamp amplifier (Physiologic Instruments, San Diego, CA) and an electronic chart recorder (PowerLab; ADInstruments, Grand Junction, CO), and the tissues were continuously short-circuited by voltage clamping to 0 mV, after fluid resistance and asymmetry voltage compensation. Changes in transepithelial resistance (\( R_T \)) were monitored and calculated with Ohm’s law from the current excursions resulting from periodic 2- or 5-mV bipolar voltage pulses. T84 cell monolayers with \( R_T \geq 1,000 \) ohm-cm\(^2\) under basal conditions were used for experimentation. Net stimulated \( I_{sc} \) reported for each experiment was calculated by subtracting the baseline \( I_{sc} \) measured before stimulation from the peak \( I_{sc} \) measured after stimulation.

For experiments in which the basolateral membrane was permeabilized with either nystatin (see Fig. 3) or *Staphylococcus aureus* \( \alpha \)-toxin (Calbiochem, San Diego, CA; see Fig. 4), the basolateral solution was replaced with a high-Na\textsuperscript{+}, high-glucuronate, low-CI\textsuperscript{−} solution to establish a mucosa-to-serosa Cl\textsuperscript{−} concentration gradient and to prevent cell swelling due to the increased basolateral Cl\textsuperscript{−} permeability under these conditions as described previously (15, 17). The basolateral bath solutions contained (in mM) \( 95 \) Na-glucuronate, \( 25 \) NaCl, \( 25 \) NaHCO\(_3\), \( 3.3 \) KH\(_2\)PO\(_4\), \( 0.8 \) K\(_2\)HPO\(_4\), 1.2 MgCl\(_2\), 10 glucose, and either 4 Ca(glucuronate)\(_2\) (Fig. 3) or 0.1 CaCl\(_2\) (Fig.
RESULTS

We previously discovered (26) that the catalytic α-subunit of AMPK interacts with the CFTR COOH-terminal tail by using independent yeast two-hybrid and biochemical ("pull-down") techniques. It was demonstrated that CFTR can serve as a substrate for AMPK-mediated phosphorylation in vitro. Importantly, exogenous coexpression of AMPK with CFTR inhibited cAMP-stimulated CFTR whole cell conductances in Xenopus oocytes, as measured with the two-electrode voltage-clamp technique. Through expression of various mutant AMPK-α subunits, it was determined that both binding of AMPK-α I to CFTR and an active kinase domain appeared to be required to confer this inhibition in oocytes. These studies raised the possibility that the interaction of AMPK with CFTR may have physiological implications. To test the relevance of the AMPK-CFTR interaction in physiologically relevant cell types, here we have examined the cellular and subcellular distribution of both proteins by immunofluorescent staining in various rat epithelial tissues and have performed functional studies in polarized T84 epithelial cell monolayers that endogenously express both proteins (3, 26).

Immunofluorescent staining to map localization of AMPK and CFTR in epithelia. We previously showed (26) that AMPK is expressed in various CFTR-expressing cell lines. Overlapping cellular and subcellular localizations of the two proteins in epithelial tissues would be expected if the interaction between AMPK and CFTR is direct and physiologically relevant in vivo. To this end, immunofluorescent staining was performed in contiguous tissue sections of various rat epithelia, including nasopharynx, submandibular gland, pancreas, and ileum (Fig. 1). To facilitate direct comparisons between AMPK-stained structures (Fig. 1, A, D, G, and J) and CFTR-stained structures (Fig. 1, B, E, H, and K), in Fig. 1 arrows indicate similar structures in the corresponding contiguous tissue sections. Light micrographs of similar hematoxylin and eosin-stained sections from the same block are also shown to aid in the identification of relevant tissue morphology (Fig. 1, C, F, I, and L). In typical fluores-
cence micrographs of rat nasopharynx revealing submucosal gland ducts and acini, CFTR staining was very distinct at the apical membranes of surface epithelial cells lining the ducts and acini (Fig. 1B). AMPK staining was also observed in these same submucosal gland structures, but it was overall more diffuse than that of CFTR yet was also prominent near the apical membranes (Fig. 1A). A control immunostain in which no primary antibodies were used is also shown (C). Hematoxylin and eosin staining of additional similar tissue sections was performed to aid in the identification of tissue morphology (F, I, and L). Sections from nasopharynx (A–C), submandibular gland (D–F), pancreas (G–I), and ileum (J–L) are shown. To facilitate direct comparisons between AMPK-stained (top) and CFTR-stained (middle) structures, arrows indicate similar structures in the corresponding contiguous tissue sections. Scale bar = 100 μm.

In all of the epithelia examined, the distribution of AMPK was generally more diffuse than that of CFTR but nevertheless displayed an apical predominance. Importantly, wherever CFTR was present, it appeared that AMPK was present (within the optical resolution of the microscope) as well, consistent with the hypothesis that the two proteins might be close enough physically to interact in vivo. It is also interesting to note that AMPK cellular expression levels, as assessed by the intensity of staining, were generally higher in the very metabolically active surface epithelial cells and generally lower in the deeper interstitial and connective tissue regions of the sections. These data demonstrating an overlapping distribution in vivo, together
with our previous binding and functional data (26), suggest that endogenous CFTR and AMPK interact with each other in polarized epithelial cells, thus forming the basis for our further investigation into the functional effects of this interaction in the polarized T84 cell model.

Pharmacological activation of AMPK inhibits endogenous CFTR-dependent currents in polarized T84 cell monolayers. Previous published studies characterized the various transport proteins that are expressed on the apical and basolateral membranes of polarized T84 cells. In T84 cell polarized monolayers studied under the conditions of these experiments, net Cl\(^-\) secretion via CFTR is responsible for almost all of the ionic conductance of the apical membrane (3). Net basolateral transport of Cl\(^-\) likely occurs via Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporters, acting in concert with the Na\(^+\)-K\(^+\)-ATPase and K\(^+\) channels to recycle Na\(^+\) and K\(^+\) across the basolateral membrane (3, 16, 18).

To test whether AMPK modulates endogenous CFTR activity in polarized T84 cells, we first used AICAR, a cell-permeant adenosine analog. AICAR becomes phosphorylated intracellularly to form ZMP, an AMP analog, which activates endogenous AMPK in vivo (10). Polarized T84 cells grown on permeable supports were pretreated for 2 h with 1 mM AICAR (or vehicle alone) to determine whether pharmacological AMPK activation could modulate endogenous CFTR-dependent \(I_{sc}\) in Ussing chambers (Fig. 2). After steady-state basal currents were measured, 4 \(\mu M\) forskolin was added to stimulate the CFTR-mediated Cl\(^-\) conductance at the apical membrane via PKA activation. To help ensure that the forskolin-stimulated apical membrane conductance was not rate limited by the basolateral membrane conductance, 2 \(\mu M\) thapsigargin, a Ca\(^{2+}\)-ATPase inhibitor, was then added to increase cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and thereby activate basolateral Ca\(^{2+}\)-activated K\(^+\) conductance (16). This forskolin- and thapsigargin-activated \(I_{sc}\) was inhibited by addition of 500–1,000 \(\mu M\) glibenclamide to the apical bath, suggesting that it was dependent on apical CFTR activity (not shown). Addition of thapsigargin alone before forskolin did not stimulate an increase in \(I_{sc}\) (not shown), consistent with previous results suggesting that there is no significant Ca\(^{2+}\)-activated apical Cl\(^-\) conductance and that apical CFTR has little basal activity in the absence of cAMP-dependent stimulation in polarized T84 cells (3, 19). AICAR, to activate endogenous AMPK, inhibited the forskolin-stimulated \(I_{sc}\) measured in either the presence or the absence of thapsigargin (by 15.9 ± 1.6% with forskolin alone (\(P < 0.001\)) and by 18.4 ± 5.6% with forskolin + thapsigargin (\(P < 0.02\); paired \(t\)-tests, \(n = 7\)) compared with controls vs. AICAR-treated paired experiments comparing the mean (±SE) stimulated \(I_{sc}\) after 4 \(\mu M\) forskolin treatment and after forskolin + 2 \(\mu M\) thapsigargin treatment (\(\Delta P < 0.001\) for forskolin, \(P < 0.02\) for forskolin + thapsigargin relative to paired controls; \(n = 7\)).

C: AMPK kinase activities (pmol min\(^{-1}\) mg lysate protein\(^{-1}\)) in control and AICAR-treated polarized T84 cells (see EXPERIMENTAL PROCEDURES for details).

Fig. 2. Short-circuit current (\(I_{sc}\)) and AMPK kinase activity measurements of polarized T84 cell monolayers with or without exposure to 1 mM 5-amino-4-imidazolecarboxamide-1-\(\beta\)-d-ribofuranoside (AICAR) for 2 h before and during the experiments to stimulate endogenous AMPK. This AICAR concentration is comparable to that used in other cell types to produce a maximal effect (10). The 2-h exposure time was determined empirically to produce the greatest effect on \(I_{sc}\) after several experimental trials with varying AICAR preincubation periods. A: \(I_{sc}\) traces from a typical paired experiment under basal conditions, after addition of 4 \(\mu M\) forskolin to the basolateral bath, and then after addition of 2 \(\mu M\) thapsigargin to the basolateral bath. Voltage pulses (±5 mV) were applied every 30 s to monitor changes in transepithelial resistance (\(R_{te}\)). B: summary of control vs. AICAR-treated paired experiments comparing the mean (±SE) stimulated \(I_{sc}\) after 4 \(\mu M\) forskolin treatment and after forskolin + 2 \(\mu M\) thapsigargin treatment (\(\Delta P < 0.001\) for forskolin, \(P < 0.02\) for forskolin + thapsigargin relative to paired controls; \(n = 7\)).
untreated paired controls (Fig. 2B). Under these conditions AICAR pretreatment stimulated endogenous AMPK kinase activity by 96% (from 0.22 ± 0.04 to 0.43 ± 0.03 pmol·min⁻¹·μg lysate protein⁻¹; *P < 0.05, *n = 2), as assessed by an in vitro kinase assay from cell lysates (Fig. 2C). Thus AICAR stimulated endogenous AMPK activity in T84 cell monolayers, and this stimulation was associated with an inhibition of endogenous CFTR-dependent currents.

AMPK-dependent inhibition of CFTR in T84 cells with basolateral membrane permeabilization. Because currents measured across intact monolayers under short-circuit conditions traverse both membranes in series, it was possible that the observed inhibition of \( I_{sc} \) by AICAR represented, at least in part, inhibition of basolateral membrane transport pathways. To examine the effects of AMPK on the apical membrane CFTR Cl⁻ conductance specifically, the basolateral membrane was permeabilized with either nystatin (Fig. 3) or *S. aureus* α-toxin (Fig. 4) to render the apical membrane conductance rate limiting. The basolateral bathing solution was substituted with a solution containing a low Cl⁻ concentration to establish a mucosa-to-serosa Cl⁻ concentration gradient as described previously (15). For the results shown in Fig. 3, T84 cell monolayers were preincubated in serum-free medium with or without the addition of 1 mM AICAR for 2 h before being mounted in Ussing chambers (time 0). Nystatin (180 μg/ml) was added to the basolateral bath at the indicated time to permeabilize the basolateral membrane to small monovalent ions. Under these conditions any changes in observed \( I_{sc} \) in response to cell activation should be directly attributable to changes in the apical membrane conductance, which is dependent on the activity of CFTR. After ~20–30 min, to allow \( I_{sc} \) to reach a steady state, 4 μM forskolin was added to the basolateral bath to stimulate apical membrane CFTR channel conductance. Under these asymmetrical conditions the Cl⁻ gradient was reversed, so activation of apical membrane CFTR conductance caused a rapid downward current deflection (Fig. 3A). A forskolin-independent stimulation was partially reversed (~50%) by subsequent 250 μM glibenclamide treatment (and more so with further glibenclamide treatments; not shown), suggesting that the current was mediated predominantly by apical CFTR. The forskolin-activated CFTR-dependent Cl⁻ current was inhibited by 24.1 ± 3.0% (P < 0.05, paired t-test, *n = 4) in the AICAR-pretreated monolayers relative to paired controls (Fig. 3B). These results are therefore in agreement with those obtained with nonpermeabilized monolayers.

As another approach to modulate AMPK activity, we modulated the intracellular [AMP]-to-[ATP] ratio by elevating the intracellular [AMP] ([AMP]ᵢ). Elevation of the [AMP]-to-[ATP] ratio enhances the activity of AMPK. To enable modulation of cytoplasmic [AMP], we permeabilized the basolateral membrane with *S. aureus* α-toxin. *S. aureus* α-toxin forms transmembrane pores that allow the passage of macromolecules up to 2–4 kDa in size (53), which thereby enabled intracellular AMP-to-ATP ratios to be varied simply by adjusting [AMP] in the basolateral bathing solution (Fig. 4). Paired T84 cell monolayers were mounted in Ussing chambers in the asymmetrical bathing solutions described above, and 250 U/ml α-toxin was added to the basolateral bath at the indicated time to permeabilize the basolateral membrane for 30 min. Na-AMP was added to the basolateral solution to modulate [AMP]ᵢ and thereby activate endogenous intracellular AMPK during this 30-min incubation period. The [AMP] used (1–5 mM) have been shown not to compete significantly with ATP at the CFTR nucleotide-binding folds (49), yet they should activate AMPK (42). The adenyl-
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...kinase inhibitor diadenosine pentaphosphate (AP5A, 100 μM; Ref. 38) was added to the basolateral solution to prevent intracellular conversion of AMP to ADP, because ADP can directly compete with ATP for binding to the nucleotide-binding domains (NBDs) of CFTR and thereby inhibit the channel (49). CFTR was then stimulated by addition of 200 μM cAMP to the basolateral bath at the indicated time, which quickly entered the cells through the pores formed by α-toxin, as evidenced by the rapid activation of the apical membrane I_{sc} (Fig. 4A). Addition of cAMP to nonpermeabilized T84 cell monolayers had no effect on I_{sc} (not shown). cAMP-stimulated CFTR-mediated Cl⁻ currents were inhibited by 36.8 ± 6.9% (P < 0.05; n = 8) in T84 cell monolayers exposed to 5 mM AMP (Fig. 4B) and by 25.5 ± 12.9% by exposure to 1 mM AMP (P = 0.07, n = 8; not shown). Under these conditions it is unclear what the local [AMP] and [ATP] were in the vicinity of CFTR and AMPK because of, for example, unstimulated layer effects inside cells. Therefore, different [AMP] were used.

Additional control experiments were performed to ensure that the observed inhibitory effects of added AMP were not due to prior AMP activation of adenosine (Ado) receptors. For example, we considered the possibility that an initial stimulation of CFTR channel activity by Ado receptor activation could blunt a subsequent stimulation of CFTR by cAMP. However, blockade of Ado receptors with the inhibitor 8-(p-sulphophenyl)-theophylline (8-SPT; 200 μM) in the presence of AMP had no effect on subsequent cAMP stimulation of apical membrane CFTR-dependent I_{sc} in α-toxin-permeabilized T84 cells compared with control cells not treated with 8-SPT (Fig. 4C). Furthermore, pretreatment with 200 μM Ado for 20–30 min had no effect on the subsequent cAMP-stimulated CFTR-dependent I_{sc} compared with control T84 cell monolayers not treated with Ado (Fig. 4D). Thus we conclude that an elevated [AMP], inhibits the activity of CFTR in T84 cell apical membranes by a mechanism(s) that does not involve direct effects on nucleotide binding by CFTR or activation of Ado receptors. Together, the results from intact monolayers and two sets of experiments using permeabilized monolayers to isolate observed effects of AMPK to CFTR specifically are consistent in suggesting that activation of endogenous AMPK by either AICAR or AMP results in the inhibition of endogenous CFTR Cl⁻ channel activity in polarized epithelial cells.

Modulation of AMPK-dependent inhibition of endogenous CFTR by mutant kinase overexpression. To manipulate AMPK activity by a nonpharmacological approach, we specifically altered its kinase activity with a dominant-negative approach. A Lys to Arg (K45R) point mutation at the active site in the kinase domain of the α-subunit of AMPK renders the kinase catalytically dead (12). Chronic overexpression of this kinase...

Fig. 4. I_{sc} measurements of polarized T84 cells after basolateral permeabilization with Staphylococcus aureus α-toxin. A: traces from a typical paired experiment comparing the addition of 5 mM Na-AMP vs. 5 mM Na-gluconate (control) to the basolateral bath, followed by basolateral permeabilization with 250 U/ml α-toxin, attainment of steady-state current, addition of 200 μM cAMP to the basolateral bath, and finally addition of 250 μM glibenclamide to the apical bath. Voltage pulses (±2 mV) were applied every 60 s to monitor changes in total R_{Isc}. AP5A, diadenosine pentaphosphate. B: mean ± SE changes in I_{sc} from paired experiments after addition of cAMP (n = 8; *P < 0.05, paired t-test). C: mean ± SE cAMP-stimulated I_{sc} in permeabilized monolayers treated as above with 5 mM Na-AMP with or without prior treatment with 200 μM 8-(p-sulfophenyl)-theophylline (8-SPT) to block Ado (Ado) receptors (n = 3; P = 0.96, paired t-test). D: mean ± SE CAMP-stimulated I_{sc} in permeabilized monolayers with or without 20- to 30-min prior stimulation by 200 μM Ado (n = 3; P = 0.16, paired t-test).
dead α-K45R mutant in cultured cells (63) and in transgenic mice (44) appears to cause a downregulation of the endogenous wild-type α-subunit through competition for binding to the endogenous regulatory β- and γ-subunits. Therefore, overexpression of the α-K45R subunit has a dominant-negative effect on AMPK activity in vivo (44). We exploited this observation by using lentiviral transduction to overexpress either the wild-type AMPK-α1 subunit or the dominant-negative AMPK-α1-K45R subunit in polarized T84 cell monolayers as a specific method to modulate the activity of AMPK (Fig. 5). The lentiviral transduction system was selected because it was shown recently to transduce intact airway epithelium in vivo with very high efficiency and stability (34). Expression of the exogenous HA-tagged AMPK-α1 subunits in the monolayers was confirmed by immunoblotting for the HA epitope tag in T84 cellular lysates obtained from the permeable supports after each Ussing chamber experiment (Fig. 5C). We hypothesized that overexpression of the dominant-negative AMPK-α1-K45R mutant might result in enhanced CFTR-mediated Cl− currents compared with wild-type AMPK-α1, if CFTR activity was tonically inhibited by the wild-type kinase. The magnitudes of the Isc activated by the combined treatment of forskolin and thapsigargin were then compared between the two groups of monolayers (Fig. 5, A and B). Compared with the wild-type AMPK-α1-transduced monolayers, the mutant AMPK-α1-K45R-transduced T84 cell monolayers exhibited a 22.4 ± 4.6% greater stimulated Isc (P < 0.01, paired t-test, n = 6). This result therefore suggests that overexpression of the dominant-negative AMPK-α1-K45R mutant in polarized T84 cells reduced the inhibition of CFTR by endogenous AMPK, i.e., inhibition of endogenous AMPK activity resulted in a disinhibition (or stimulation) of CFTR. Because control experiments suggested that only ~50% of the T84 cells in the monolayer under these conditions expressed the exogenous protein (Fig. 5D), it is likely that this result underestimates the true magnitude of the dominant-negative effect.

**DISCUSSION**

The maintenance of ionic gradients across the plasma membrane by various cellular pump and leak pathways is vital in general for normal cellular functioning and in particular for the coordinated transport of solutes and fluids across polarized epithelia. Cellular transport processes consume a substantial proportion

![Fig. 5. Forskolin + thapsigargin-stimulated Isc in polarized T84 cell monolayers overexpressing wild-type (wt) AMPK-α1 or dominant-negative AMPK-α1-K45R.](http://ajpcell.physiology.org/)

A: traces from a typical paired experiment before and after addition of 4 μM forskolin to the basolateral bath followed by addition of 2 μM thapsigargin to the basolateral bath. Voltage pulses (±2 mV) were applied every 60 s to monitor changes in Rcy. B: mean ± SE changes in Isc from paired experiments after addition of forskolin + thapsigargin (n = 6; *P < 0.01, paired t-test). These mean measured Isc values are in the same range as other control (nontransduced) CFTR currents that were measured at about the same time in other experiments. C: representative anti-hemagglutinin (HA) immunoblot of 75–100 μg of lysate protein from HA-AMPK-α1-wt- and HA-AMPK-α1-K45R-transduced T84 cell monolayers, along with nontransduced control to confirm expression of the exogenous HA-tagged AMPK-α1 subunits (see EXPERIMENTAL PROCEDURES for details). D: fluorescent micrograph of T84 cell monolayer transduced with a vesicular stomatitis virus G (VSV-G)-pseudotyped green fluorescent protein (GFP)-expressing lentiviral construct at a similar titer to that used for AMPK transduction above under the same conditions (see EXPERIMENTAL PROCEDURES for details).
of total cellular metabolic energy and thus would be expected to respond closely to changes in cellular metabolic status. Our recent discovery of a functional interaction between AMPK and CFTR suggests a novel mechanism for such coupling between ion transport and cellular metabolism. The results obtained in the present study now suggest that the AMPK-CFTR interaction is physiologically relevant in polarized epithelia.

AMPK and CFTR share an overlapping apical distribution in various rat epithelial tissues (Fig. 1). Previous yeast two-hybrid and biochemical techniques demonstrated that the COOH-terminal regulatory domain of the AMPK-α1 catalytic subunit binds to the COOH-terminal tail of CFTR (26). The overlapping localization of the two proteins in epithelial cells may suggest that the proteins do indeed interact at the apical plasma membrane and, more speculatively, that the apical localization of AMPK is a consequence of its interaction with CFTR. However, because AMPK exhibits a more diffuse subcellular distribution compared with CFTR, it is clear that binding to CFTR cannot be the sole determinant of AMPK localization. Indeed, the AMPK β-subunit has been shown to be myristoylated (41), and this modification may generally promote localization of the AMPK holoenzyme to membranes. Localization of the kinase to the apical membrane might enable AMPK to effectively and directly modulate the function of CFTR as well as other nearby membrane proteins. Future studies of AMPK localization, using epithelial tissues from CFTR knockout mice or CF patients, may provide insights into the possible role of CFTR as an AMPK-anchoring protein.

In our examination of AMPK immunolocalization, it appeared that AMPK cellular expression levels, as assessed by the intensity of staining, were high in the very metabolically active surface epithelial cells and generally lower in the deeper interstitial and connective tissue regions of the tissue sections (Fig. 1). Epithelial cells generally expend a large percentage of total cellular metabolic energy on membrane transport processes, and their metabolic consumption rate is highly correlated with their rate of active ion transport (39). The fact that cellular expression levels of this metabolic-sensing kinase appear to correlate with cellular metabolic activity in epithelial tissues is perhaps not surprising because previous studies demonstrated increased AMPK expression in highly metabolically active cells in other tissues, such as neurons in the brain (13) and ventricular myocytes in rat hearts with pressure-overload hypertrophy (57).

The immunolocalization studies indicate that the cellular localizations of AMPK and CFTR overlap, but they provide no direct evidence that the proteins interact or that the interaction has physiological relevance. In the present study, we used several independent functional approaches to modulate endogenous AMPK activity and then determined the effects on endogenous CFTR-mediated Cl− currents in polarized T84 cell monolayers. The T84 cell line is a widely used model of epithelial Cl− secretion because it forms polarized, high-resistance monolayers and expresses relevant transport proteins in its apical and basolateral membranes (18). Of relevance for the present studies, the Iec through T84 monolayers is conducted entirely by CFTR at the apical membrane. Pretreatment with the pharmacological AMPK activator AICAR inhibited the magnitude of stimulated CFTR-dependent Iec in both intact T84 cell monolayers (Fig. 2) and nystatin-permeabilized monolayers, in which the effects could be specifically localized to CFTR at the apical membrane (Fig. 3). Furthermore, elevating intracellular [AMP] directly through α-toxin permeabilization of the basolateral membrane and dialysis into the cells of high concentrations of AMP from the basolateral bath also inhibited CFTR-mediated Cl− currents (Fig. 4). The latter effect could not be explained by activation of adenosine receptors (Fig. 4, C and D), and it is unlikely to be due to a direct effect of AMP on CFTR, because no AMP-dependent effects on CFTR single-channel gating have been observed at concentrations up to 3 mM (49). The AMP-dependent inhibition of CFTR activity was also unlikely to be caused by intracellular conversion of AMP and ATP to ADP through adenylate kinase, because the adenylate kinase inhibitor AP5A was present throughout the experiments. Thus the [AMP]i and [ADP]i should have been relatively unaffected by intracellular dialysis of AMP, so the inhibition observed with AMP treatment under these conditions cannot be attributed to a reduction of [ATP]i or to an elevation of [AMP]i and therefore intracellular [AMP]/[ATP] ratio) inhibited CFTR by directly activating endogenous intracellular AMPK. Finally, overexpression of a kinase-dead, dominant-negative AMPK mutant in the polarized T84 cell monolayers enhanced CFTR-mediated transepithelial Cl− currents (Fig. 5). This result provides specific evidence that AMPK can modulate CFTR activity in a relevant epithelial system. The enhancement of CFTR-dependent Iec seen after suppression of endogenous AMPK activity with the dominant-negative mutant suggests that there may be a tonic level of AMPK activity in these T84 cells under the conditions of these experiments that inhibited CFTR activity. Indeed, cultured cells, including epithelial cell lines, are chronically hypoxic when cultured submerged in medium (2), as the T84 cells were for these studies, which is a condition that would promote basal AMPK activity. By the dominant-negative downregulation of this AMPK activity, a disinhibition (or enhancement) of CFTR Iec could be observed. It is also possible that overexpression of the wild-type AMPK α1-subunit promoted endogenous AMPK activity and thereby inhibited CFTR in the control group. For this to have occurred, however, sufficient amounts of endogenous regulatory β- and γ-subunits would need to be available to associate with the expressed α-subunit, because they are required to confer full AMPK activity.

The data presented here provide the first compelling evidence for a physiologically relevant regulation of an
ion channel (CFTR) by AMPK. AMPK activity may also be important in the regulation of other membrane transport proteins in vivo. It has been long observed that a loss in membrane transport protein activity, expression, and polarity is associated with ischemic injury to epithelial tissues [e.g., in the kidney (25)], although the mechanisms underlying this effect are unclear. For example, hypoxia and cellular energy depletion in epithelial tissues inhibit ATP-dependent membrane transporters such as the Na\(^{+}\)-K\(^{+}\)-ATPase and P-glycoprotein, also known as multidrug resistance protein (MDR) (6, 39). Pertinent to this study, Bell and Quinton (4) previously measured Cl\(^-\) conductance in α-toxin-permeabilized T84 cells before and after metabolic depletion. It was concluded that a non-hyloidal, presumably direct, interaction of ATP with CFTR was involved in the coupling between cellular metabolic status and CFTR activity (4). A direct ATP-CFTR interaction certainly occurs through ATP binding to the NBDs of CFTR. However, it is unlikely that small changes in [ATP] would be sufficient to tightly couple CFTR activity to cellular metabolic state by this mechanism under normal physiological conditions because ambient cellular [ATP] are in the millimolar range, which is generally much higher than the saturating concentrations for ATP binding to the NBDs of CFTR (1). Similarly, it has been noted that only modest decreases in cellular [ATP], remaining within the millimolar range, dramatically inhibit the activities of the Na\(^{+}\)-K\(^{+}\)-ATPase and MDR, despite their having affinities for ATP of <100 μM. This discrepancy has been rationalized by proposals that large gradients of [ATP] exist in cells (6). We suggest that AMPK may provide a more sensitive means of coupling changes in the cellular metabolic state to transporter activity. Relatively small changes in intracellular ATP levels result in large changes in the intracellular AMP-to-ATP ratio because of the rapid interconversion of ATP, ADP, and AMP by adenylyl kinase (see Ref. 30 for review). Because AMPK activity is sensitive to the AMP-to-ATP ratio, AMPK can serve as a primary sensor of (even minor) changes in metabolic state and may therefore play an important role in the initiation of cellular events that occur both during physiological fluctuations of [ATP] and during pathological reductions in [ATP] (i.e., in response to ischemic or hypoxic injury). Recently, mutations in the gene encoding the AMPK-γ regulatory subunit (PRKAG2) in cardiac muscle were shown to be responsible for familial cases of ventricular preexcitation arrhythmias (Wolff-Parkinson-White syndrome), which led to the suggestion that AMPK may be an important regulator of ion channels in the heart (23, 24). Of note, CFTR is expressed in heart muscle (31) and has been postulated to play a role in the genesis of cardiac arrhythmias (32). Also, ATP-sensitive K\(^+\) (K\(\text{ATP}\)) channels, which are composed of inward rectifying K\(^+\) (K\(_{\text{ir}}\)) subunits coupled stoichiometrically with the sulfonylurea receptor SUR (51), another ABC transporter closely related to CFTR, are regulated by changes in cellular metabolic state and are important ion channels found in the heart (22).

Further studies are thus warranted to investigate the potential role of AMPK in the modulation of membrane transport proteins, which could lead to new insights into the pathogenesis and treatment of many disorders, including ischemic tissue injury and cardiac arrhythmias.

In summary, we have demonstrated that endogenous AMPK and CFTR share an apical membrane localization in epithelial tissues and that modulating endogenous AMPK activity in epithelial cells regulates the activity of CFTR. The AMPK-CFTR interaction may constitute a novel mechanism to link CFTR ion channel activity to cell metabolic status, thereby coupling cellular metabolism with transepithelial solute transport and the maintenance of cellular ion gradients.

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