Inhibition of the KCa3.1 channels by AMP-activated protein kinase in human airway epithelial cells

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Submitted 12 August 2008; accepted in final form 1 December 2008


First published December 3, 2008; doi:10.1152/ajpcell.00418.2008.

The vectorial transport of ions and water across epithelial cells depends to a large extent on the coordination of the apical and basolateral ion fluxes with energy supply. In this work we provide the first evidence for a regulation by the 5′-AMP-activated protein kinase (AMPK) of the calcium-activated potassium channel KCa3.1 expressed at the basolateral membrane of a large variety of epithelial cells. Inside-out patch-clamp experiments performed on human embryonic kidney (HEK) cells stably transfected with KCa3.1 first revealed a decrease in KCa3.1 activity following the internal addition of AMP at a fixed ATP concentration. This effect was dose dependent with half inhibition at 140 μM AMP in 1 mM ATP. Evidence for an interaction between the COOH-terminal region of KCa3.1 and the γ1-subunit of AMPK was next obtained by two-hybrid screening and pull-down experiments. Our two-hybrid analysis confirmed in addition that the amino acids extending from Asp190 to Ala200 in COOH-terminal were essential for the interaction AMPK-γ1/KCa3.1. Inside-out experiments on cells coexpressing KCa3.1 with the dominant negative AMPK-γ1-R299G mutant showed a reduced sensitivity of KCa3.1 to AMP, arguing for a functional link between KCa3.1 and the γ1-subunit of AMPK. More importantly, coimmunoprecipitation experiments carried out on bronchial epithelial NuLi cells provided direct evidence for the formation of a KCa3.1/AMPK-γ1 complex at endogenous AMPK and KCa3.1 expression levels. Finally, treating NuLi monolayers with the membrane permeant AMPK activator 5-aminoimidazole-4-carboxamide-1-D-ribofuranoside (AICAR) caused a significant decrease of the KCa3.1-mediated short-circuit currents, an effect reversible by coincubation with the AMPK inhibitor Compound C. These observations argue for a regulation of KCa3.1 by AMPK in a functional epithelium through protein/protein interactions involving the γ1-subunit of AMPK.

potassium channel; protein-protein interactions; cystic fibrosis

5′-AMP-ACTIVATED PROTEIN KINASE (AMPK) is an ubiquitously expressed metabolic-sensing Ser/Thr kinase that plays a key role in the energy homeostasis of cells (26). AMPK exists as a heterotrimer constituted of a catalytic α-subunit with two regulatory subunits β and γ encoded by distinct genes (α1, α2, β1, β2, γ1, γ2, γ3). AMPK activity is largely controlled by upstream kinases such as LKB1 and calmodulin-dependent protein kinase kinase (CaMKKβ), which phosphorylate the AMPK-α subunit at Thr172 (21). It was recently reported that AMP contributes to maintain the AMPK-α subunit in an active phosphorylated state by inhibiting AMPK dephosphorylation at Thr172 (19, 20, 36). AMP can also induce an allosteric conformational change of the α-subunit leading to an increase in kinase activity (19, 20, 22, 23). Several lines of evidence point to the γ-subunit as the major site for AMP allosteric control of AMPK (9). The solved structure of the mammalian AMPK indeed confirmed the presence of two exchangeable AMP/ATP sites on opposite sides of the γ-subunit (49), in accordance with a control of the AMPK activity determined by the AMP-to-ATP ratio (19).

There is now strong evidence that AMPK plays a prominent role in coupling the transepithelial transport of ions and water in several epithelium preparations to the metabolic state of the cells. AMPK was found, for instance, to bind to the COOH-terminal tail of fibrosis transmembrane conductance regulator (CFTR) and decrease the channel open probability (17). AMPK activation was similarly reported to decrease epithelial Na channel (ENaC) currents by downregulating the number of active channels at the plasma membrane thus reducing excessive salt and water reabsorption in metabolic stress conditions (4, 7, 15). This effect was subsequently attributed to an increase by AMPK of the Nedd4-2-dependent ENaC retrieval from the plasma membrane. In addition to CFTR and ENaC, the vectorial transport of ions in airway epithelial cells also depends on the activation of K+ channels, including the Ca2+-activated K+ channel of intermediate conductance KCa3.1 also known as KCNN4, IK1, or SK4. This channel consists in a tetrameric protein with each subunit organized in six transmembrane segments. The channel Ca2+-sensitivity is conferred by the Ca2+-binding protein calmodulin (CaM), which is constitutively bound to KCa3.1 in the COOH-terminus (31). KCa3.1 activation by potentiators such as 1-ethyl-2-benzimidazolone (EBIO) and 4-chloro-benzo[F] isouquinoline (CBIQ) was found to stimulate Cl− secretion and Na+ absorption in several epithelial cell preparations, including colonic epithelia, T84, Calu-3, and human bronchial cells (10, 30, 40, 43, 45). These observations led to conclude that KCa3.1 channels could play a prominent role in transepithelial transport by establishing a suitable driving force to maintain a sustained Cl− efflux and Na+ influx at the apical membrane of epithelial cells (3, 12, 32). KCa3.1 is also regulated by ATP, suggesting a potential link between metabolic stress and...
KCa3.1 activity. Recent studies have shown that internal ATP stimulates the human KCa3.1 channel activity through the phosphorylation by the nucleoside diphosphate kinase NDPK-B of a histidine residue located within the channel COOH-terminal region (42). The action of ATP required Ca\(^{2+}\) and resulted in an apparent greater Ca\(^{2+}\) sensitivity (41). PKA has also been documented to regulate the human KCa3.1 channel, but it is currently believed that KCa3.1 is not itself a target of PKA because mutations of the PKA-consensus sites in the human KCa3.1 affected neither the basal nor the ATP-activated current (13, 14). Altogether, these observations point toward a complex ATP-dependent regulation of the KCa3.1 channel activity that could involve multiple intracellular sites and/or several ATP-sensitive auxiliary proteins.

Despite the importance of KCa3.1 in modulating Cl\(^{-}\) efflux and Na\(^{+}\) influx in epithelial cells, there is currently no evidence for KCa3.1 being regulated by AMPK. In this work, we show a downregulation of KCa3.1 activity in response to an increase in AMP concentration, an effect we interpret as coming from an interaction between the COOH-terminal region of KCa3.1 and the AMP-binding \(\gamma\)-subunit of AMPK. Therefore, our results point toward a global modification by AMPK of the ion transport properties in Cl\(^{-}\)-secreting epithelia not exclusively mediated by CFTR and ENaC but that also includes a contribution of the KCa3.1 channel.

**MATERIALS AND METHODS**

**Cell cultures.** HEK cells were cultured in Dulbecco's high-glucose minimum essential medium (DMEM-HG) supplemented with 2.2g/l NaHCO\(_3\), 10% FBS, penicillin, and streptomycin under 5% CO\(_2\) atmosphere at 37°C. HEK-293 cells transfected with the KCa3.1 channel tagged in COOH-terminal with the Myc epitope (HIK cells) were kindly supplied by Dr. Daniel C. Devor, University of Pittsburgh, and grown in the presence of 0.1mg/ml zeocin. Hemagglutinin (HA)-tagged KCa3.1 channels (HA-KCa3.1) were generated by inserting the HA epitope tag (YPYDVPDYA) into the channel second extracellular loop between G132 and A133 as described elsewhere (25). HA-KCa3.1 cells were obtained by transfecting HEK-293 cells with HA-KCa3.1 cDNA cloned into the pCMV-Tag5 vector (Stratagene, LaJolla, CA) using Lipofectamine 2000 (Invitrogen, Burlington, ON). Similarly, HIK-AMPK-\(\gamma\)-R299G cells were obtained by transfecting HIK cells with Lipofectamine 2000 with AMPK-\(\gamma\)-R299G cloned in pCMV-Tag5. Stable cell lines were generated by antibiotic selection 48 h posttransfection (0.4 mg/ml G418 for HIK-KCa3.1 and 0.4 mg/ml G418 plus 0.1 mg/ml zeocin for HIK+AMPK-\(\gamma\)-R299G cells). Selection was typically complete within 14 days after transfection.

For immunofluorescence, HA-KCa3.1 cells were seeded at 10\(^5\) cells on poly-L-lysine hydrobromide (Sigma-Aldrich, Oakville, ON)-coated microscope cover glass (22 mm diameter, Fisher Scientific, Ottawa, ON) and grown in 35-mm plates for 4 days. An identical procedure was applied for HIK cells used in patch-clamp experiments.

Bronchial epithelial NuLi-1 (Normal Lung) cells were kindly provided by Dr. J. Zabner, University of Iowa. The NuLi-1 line was obtained by immortalization of human airway epithelia of normal genotype, with a reverse transcriptase component of telomerase (hTERT) and human papillomavirus type 16 (HPV-16) E6 and E7 genes (50). The cells were cultured from passages 11 up to 17 on plastic support in presence of bronchial epithelial cell growth medium (BEGM) supplemented with hydrocortisone, bovine pituitary extract, epidermal growth factor (EGF), transferrin, bovine insulin, triiodothyronine, epinephrine, and retinoic acid (Cambrex Biosciences, Walkerville, MD). For electrophysiological experiments, NuLi cells were grown on Costar Transwell permeant filters (Costar Transwell, Toronto, ON) at the air-liquid interface in DMEM-F12 medium (Invitrogen) supplemented with UltraSerG (Biosera SPA, Cergy-Saint-Christophe, France), which enhances ion transport (50). During the culture procedure the medium coming from the basolateral side, which appeared at the apical surface, was removed every 2–3 days, and fresh medium was added at the basolateral side. After 4 to 6 wk of culture, the formation of an air-liquid interface (luminal face exposed to air while the basalolateral side was bathed in culture medium) was taken as an indication that NuLi monolayers were confluent and could form a polarized, differentiated bronchial epithelium. The mean resistances (R) and short-circuit currents (Isc) measured in Ussing chamber were under those conditions equal to 813 ± 92 Ω-cm\(^{2}\) and 25 ± 2 μA/cm\(^{2}\) (n = 23), respectively. These values are similar to that reported previously by Zabner et al. (52).

**Bacteriophage two-ybrid analysis.** Two-ybrid analysis was performed using the BacterioMatch II Two-Hybrid System (Stratagene). The COOH-terminal segment of KCa3.1 extending from Leu\(^{330}\) to Ala\(^^{414}\), which was used as the bait, was inserted in the frame in the pB7T vector and tested against a cDNA HeLa library cloned into pTRG vector. The choice of the library was based on previous study which showed that KCa3.1 is highly expressed in HeLa cells (37). Transfomants (1–2 × 10\(^{7}\)) from HeLa cDNA library were screened, and the colonies were obtained from the initial screening plates enriched on a second selective plate. Putative positive colonies were validated using streptomycin resistance as a secondary reporter. The nucleotide sequence of the target DNA was determined (BioS&T, Lachine, Quebec, Canada) and tested against nucleotide sequence databases to identify related proteins. We verified the specificity of the interactions by one-on-one two-ybrid analysis and cotransformed the reporter strain using each purified target plasmid paired with the recombinant pB7T plasmid.

**Purification of recombinant GST-KCa3.1 fusion protein and affinity pull-down assays with immobilized GST fusion proteins.** A cDNA construct encoding the COOH-terminal glutathione S-transferase GST-KCa3.1 fusion protein was created by subcloning the human KCa3.1 cDNA (from Arg\(^{330}\) to Lys\(^{427}\)) into the GST fusion protein vector pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ). After sequencing was completed, GST and GST-KCa3.1 constructs were transformed into BL21(DE3) bacteria competent cells (Novagen, San Diego, CA), induced with isopropyl-1-thio- D-galactopyranoside (IPTG 0.2 mM) for 3 h, and lysed according to MagneGST Protein kit (Promega). The quality of the fusion proteins was assessed with Coomassie staining and tested by Western blot analysis using a rabbit anti-KCa3.1 antibody (1:200, Sigma) that was detected with horse-radish peroxidase-conjugated goat anti-rabbit IgG (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA) as secondary antibody.

For the affinity pull-down assay, GST-KCa3.1 fusion proteins (from Arg\(^{330}\) to Lys\(^{427}\)) and GST alone (50 μg each) were incubated overnight at 4°C in the presence of 1 μg of HIK cell lysates. Lysates were obtained by vortexing cells in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, plus 0.5% SDS complete protease inhibitor mixture (Roche Applied Science, Laval, Quebec, Canada), and centrifuged at 100,000 g for 45 min at 4°C to prepare a high-speed supernatant. Proteins from HIK cell lysates purified as described above were incubated onto MagneGST-COOH-terminal KCa3.1 particles overnight at 4°C, washed twice in a phosphate buffer containing (in mM) 4.2 Na\(_2\)HPO\(_4\), 2 KH\(_2\)PO\(_4\), 140 NaCl, and 10 KCl, and eluted with 2× SDS sample buffer for 3 min at 85°C. AMPK-\(\gamma\)-R299G subunit pulled down by the GST-KCa3.1 fusion protein were detected by Western blot analysis. After protein transfer to nitrocellulose membrane (Hybond, Amersham Biosciences), the membrane was blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 and incubated overnight at 4°C with rabbit antibody anti-AMPK-\(\gamma\) antibody (1:2,000, Invitrogen). The membrane was then washed three times with TBS containing 0.1% Tween, and the secondary antibody (horse-radish peroxidase-conjugated goat anti-rabbit IgG; Jackson Immuno-
Research Laboratories) was added at 1:20,000 dilution for 1 h at room temperature. Immunoreactive bands were visualized using ECL detection reagent (Pierce, Rockford, IL) and exposed on X-ray films.

**Coimmunoprecipitation of KCa3.1 and γ1-subunit of AMPK from NuLi cells.** Lysis of NuLi cells was performed with buffer I (150 mM NaCl, 50 mM Tris·HCl, pH 7.5, 1% Nonidet P40, and 0.5% sodium deoxycholate) from the immunoprecipitation kit (protein A, Roche Applied Science) following the manufacturer’s instructions. The lysates were homogenized and isolated by centrifugation as described previously. After quantification using the Bradford method, 1–2 mg soluble lysate was precleared with 50 μl of 50% protein A-agarose suspension. For immunoprecipitation of endogenous KCa3.1 or AMPK-γ1 proteins, precleared soluble lysates were incubated for 1–2 h with a rabbit anti-KCa3.1 antibody (1:100, Alomone Labs, Jerusalem, Israel) or with a rabbit anti-AMPK-γ1 antibody (1:100 or 1:150, Abcam, Cambridge, MA). The immunocomplexes were precipitated by incubating overnight at 4°C with 50 μl of 50% protein A-agarose suspension. After being washed twice with buffer I and buffer 2 (high salt, 500 mM NaCl, 50 mM Tris·HCl, pH 7.5, 0.1% Nonidet P40, 0.05% sodium deoxycholate) and 10 mM Tris·HCl, pH 7.5, 0.1% Nonidet P40, 0.05% sodium deoxycholate), proteins bound to beads were collected by centrifugation and eluted with 30–50 μl of 2X sample buffer as described for the pull-down assays at 95°C for 5 min. The immunoprecipitated proteins were resolved on a 12% SDS-PAGE gel and transferred to PVDF membranes (Molecular Device, Union City, CA) at a sampling rate of 2 kHz with the QuB software (35). The pipette solution contained (in mM) 145 K-glutamate, 5 KCl, 2.5 MgCl₂, 1 EGTA, and 10 HEPES adjusted at pH 7.4 with KOH. The bathing solution consisted of (in mM) 145 K-glutamate, 5 KCl, 2.5 MgCl₂, 10 HEPES, and 1 EGTA plus CaCl₂ to yield a final free Ca²⁺ concentration of 10 μM at pH 7.4. When using ATP-containing bath solutions, the free Ca²⁺ and Mg²⁺ concentrations were adjusted to remain constant at 10 μM and 2.5 mM, respectively. The free Ca²⁺ concentration for each solution was calculated using the EQCAL multiple equilibrium software (Biosoft, Cambridge, UK). To obtain a Ca²⁺-free bath solution, EGTA (1 mM) was added without CaCl₂ (estimated free Ca²⁺ <10 nM). Bath solution changes were performed as described previously using a RSC-160 rapid solution changer system (BioLogic, Grenoble, France) (35). The solution exchange time was estimated to be <20 ms (27). AMP and ATP were purchased from Sigma-Aldrich. Experiments were performed at room temperature (23°C).

**Using chamber measurements on NuLi monolayers.** NuLi epithelial cell monolayers, cultured on filters (4 cm², Costar Transwell) at air-liquid interface for 4–6 wk, were pretreated for 1–2 h (at apical and basolateral sides) with or without 5-aminoimidazole-4-carboxamido-1-β-D-ribofuranoside (AICAR, 1 mM, Sigma-Aldrich), or AICAR plus Compound-C (10 μM, Merck Frosst, Whitehouse Station, NJ) in DMEM-F12. The treated monolayer was then mounted in a heated (37°C) Ussing chamber for short-circuit current measurements. KCa3.1 stimulation was induced by bath application of 1-EBIO (Tocris, Ellisville, MO), whereas TRAM-34 (Sigma-Aldrich) was used as a specific KCa3.1 inhibitor. Total Iᵥ (Iᵥbase, Iᵥ-1-EBIO, and Iᵥ-1-EBIO-s⁻)-sensitive currents measured under our experimental conditions correspond to KCa3.1 (24, 25, 44). A series of inside-out patch-clamp experiments was next performed to determine whether the KCa3.1 activity could be regulated by AMP in these experiments, channel activity was measured at fixed Ca²⁺ (10 μM) and ATP (1 mM) concentrations. An example of inside-out patch-clamp recording illustrating the action of 200 μM AMP applied internally is presented in Fig. 1B. In most of these experiments, excision of the patch membrane resulted in a time-dependent decrease in channel activity that was reversed by the addition of 1 mM ATP to the internal medium. This effect corresponds to the well-documented stimulatory action of ATP on the KCa3.1 channel (13) and likely

**RESULTS**

**AMP inhibits recombinant KCa3.1 channel activity in inside-out.** Functional KCa3.1 channel expression in HIK cells was first confirmed in inside-out patch-clamp experiments where channel activity was measured before and after addition of the KCa3.1 inhibitor Tram-34 (Fig. 1A). The internal addition of Tram-34 (1 μM) resulted in a near total inhibition of channel activity that was slowly reversible confirming that the Ca²⁺-sensitive currents measured under our experimental conditions correspond to KCa3.1 (24, 25, 44). A series of inside-out patch-clamp experiments was next performed to determine whether the KCa3.1 activity could be regulated by AMP in these experiments, channel activity was measured at fixed Ca²⁺ and ATP (1 mM) concentrations. An example of inside-out patch-clamp recording illustrating the action of 200 μM AMP applied internally is presented in Fig. 1B. In most of these experiments, excision of the patch membrane resulted in a time-dependent decrease in channel activity that was reversed by the addition of 1 mM ATP to the internal medium. This effect corresponds to the well-documented stimulatory action of ATP on the KCa3.1 channel (13) and likely
Fig. 1. Inside-out experiments illustrating a regulation of the KCa3.1 activity by AMP. Inside-out patch-clamp recording performed on human epithelia kidney HEK-293 cells expressing the Myc-KCa3.1 channel. Recording obtained in 10 μM internal Ca^{2+} conditions at an applied membrane potential of −60 mV. Perfusion with a 0 Ca^{2+} solution (EGTA) is represented as a filled rectangle. The symbol c refers to the zero current level. A: internal addition of the KCa3.1-specific inhibitor Tram-34 (1 μM) caused a strong current inhibition that was slowly reversible, confirming that the Ca^{2+}-sensitive current measured under our experimental conditions corresponds to KCa3.1. Inset, single-channel events, confirming current jumps of 2.4 pA for an unitary conductance of 40 pS as expected for KCa3.1. B: initial addition of 1 mM ATP to the internal medium caused an increase in channel activity to reach a stable current level. Perfusion with a solution containing 1 mM ATP plus 200 μM AMP led to a 46 ± 18% (n = 17) decrease in channel activity that was reversible following the washout of AMP. C: effect of AMP on channel activity in the absence of ATP. Perfusion with an internal solution containing AMP (200 μM) in the absence of ATP did not affect the time course of the channel rundown process. This result supports an effect of AMP on KCa3.1 that is ATP dependent. D: frequency histogram of the percentage of current inhibition measured at 200 μM AMP obtained from 17 different cells. E: percentage of current inhibition measured as a function of the AMP concentration for ATP at 1 mM. The AMP concentration for half inhibition was estimated at 140 μM. Each data point represents the mean ± SD of at least 3 different experiments.

[Image: C288.png]

Involves phosphorylation of KCa3.1 by the NDPK-B protein (42). The addition of 200 μM AMP in 1 mM ATP conditions caused a 46 ± 18% (n = 17) decrease in channel activity that could be partly reversed by AMP washout. Control patch-clamp experiments were also carried out to determine whether the observed inhibitory action of AMP requires the presence of ATP. As seen, patch excision in the absence of ATP resulted in a slow rundown of the channel activity typically of the order of 45% over a 1-min period (Fig. 1C). The current recording presented in Fig. 1C indicates, however, that the rundown process was not affected by the addition of 200 μM AMP in contrast to the fast AMP response illustrated in Fig. 1B. This recording also shows that channel activity could be restored by perfusing with solution containing 1 mM ATP. These observations therefore support a model whereby the KCa3.1 channel inhibition mediated by AMP is ATP dependent. Figure 1D summarizes the results obtained from 17 different cells where AMP was applied at 200 μM with ATP at 1 mM. As seen, 10 of the 17 cells studied showed a percentage of inhibition between 40% and 60%, whereas <5% of the cells studied (1/17) responded to the addition of AMP by an inhibition of <20%. The action of AMP on channel activity was dose dependent for concentrations within the physiological range expected for AMP (Fig. 1E), with a concentration for 50% inhibition estimated at 140 μM. This result is in line with the value reported from biochemical assays, where AMPK stimulation was measured under similar AMP-to-ATP ratios (23).

**Interactions between the γ1-subunit of AMPK with the COOH-terminal (CT) domain of KCa3.1.** A regulation of the KCa3.1 channel activity by internal AMP suggests a potential control by AMPK. To establish whether such a regulatory mechanism involves a direct interaction KCa3.1/AMPK or the recruitment of an auxiliary protein, we screened a bacterial two-hybrid library for proteins that could act as KCa3.1 channel regulators through interactions with the channel COOH-terminal domain (Leu345-Ala400). Thirty positive clones were initially identified, of which nine remained positive following streptomycin-based screening. Out of these 9 clones, 3 were identified after sequencing as the human γ1-subunit of AMPK. The interaction between the KCa3.1/CT fragment (Leu345-Ala400) and the γ1-subunit of AMPK was further confirmed using the entire KCa3.1/CT (Arg287-Lys427) domain. Our result showed that this domain could also bind the γ1-subunit of AMPK (Table 1). No interaction between KCa3.1/CT and the γ1-subunit of AMPK could however be detected with KCa3.1/CT (Leu345, Asp380) fragment, indicating that the amino acids extending from
Table 1. Interaction between the COOH-terminal domain of KCa3.1 and the γ1-subunit of AMPK revealed by two-hybrid analysis

<table>
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<tr>
<th>Bait Plasmid (pBT)</th>
<th>Prey Plasmid (pTRG)</th>
<th>Control</th>
<th>AMPK-γ1</th>
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<td>KCa3.1/CT (Arg287-Lys427)</td>
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<td>KCa3.1/CT (Leu345-Ala400)</td>
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Positive two-hybrid responses were observed using the COOH-terminal (CT) fragments of KCa3.1 extending either from Arg287 to Lys427 or from Leu345 to Ala400. No interaction could, however, be detected using the fragment Leu345 to Asp380, indicating that the amino acids extending from Asp380 to Ala400 are essential for the interaction between KCa3.1 and the γ1-subunit of 5'-AMP protein kinase (AMPK). Detection of protein-protein interactions was based on transcriptional activation of the HIS3 reporter gene, which allows growth in the presence of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3 enzyme. Positives are verified by using the aadA gene, which confers streptomycin resistance, as a secondary reporter (+).

Asp380 to Ala400 are essential to the interaction between the COOH-terminal region of KCa3.1 and the AMPK-γ1 subunit. As this 21 amino acid stretch contains a consensus sequence for a leucine zipper motif (L378ydLK386ssHrL392-ekqD*L399), our results suggest that the γ1-subunit of AMPK can bind to the COOH-terminal end of KCa3.1 through a leucine zipper ([L-x(6)-L-x(6)-L]-based mechanism. GST pull-down experiments were next performed to confirm that the COOH-terminal domain of the KCa3.1 interacts with the γ1-subunit of AMPK. The overall quality and purity of the GST/GST-KCa3.1 proteins were confirmed by Coomassie staining as seen in Fig. 2A. Western blotting with an anti-KCa3.1 antibody revealed in addition a single band at the expected molecular mass of 40 kDa for the GST-KCa3.1 terminal fusion protein (Arg380 to Lys427) (Fig. 2B, lane 2), while no band was detected for GST alone (Fig. 2B, lane 1). Binding between the GST/KCa3.1 fusion protein containing the Arg380-Lys427 domain of KCa3.1 and the γ1-subunits of AMPK is demonstrated in the Western blots presented in Fig. 2C, lane 3. As seen, a band of 38 kDa corresponding to the expected molecular mass for the γ1-subunit of AMPK was detected in the total cell lysate (lane 1) and purified GST-KCa3.1 preparation (lane 3) using an anti-AMPK-γ1 antibody. No binding was detected with GST alone (lane 2). Altogether these observations strongly argue for AMPK interacting as a complex with the COOH-terminus of KCa3.1 extending from Arg380 to Lys427.

AMPK-γ1 and KCa3.1 colocalize at the plasma membrane of HEK-293 cells expressing HA-KCa3.1. Functional protein/protein interactions with KCa3.1 require that the auxiliary protein be localized at the plasma membrane. Immunofluorescence experiments were thus performed using HEK cells transfected with KCa3.1 containing an external HA tag at position G132. This approach was found to yield a better signal in immunofluorescence experiments compared with HEK cells transfected with KCa3.1 bearing a Myc tag. Comparing the HA-KCa3.1 and endogenous AMPK-γ1 labeling in Fig. 3 demonstrates that AMPK-γ1 is highly expressed at the plasma membrane and colocalized at the plasma membrane with HA-KCa3.1. There was no detectable labeling with the AlexaFluor488 and AlexaFluor594 probes in control experiments where cells were incubated in the absence of anti-HA/AMPK-γ1 primary antibodies (data not shown). Altogether, the colocalization of KCa3.1 and AMPK-γ1 at the plasma membrane suggests a membrane-delimited interaction between AMPK-γ1 and KCa3.1.

Functional AMPK-γ1 is essential for the regulation of KCa3.1 by AMP. To manipulate AMPK-γ1 activity by a nonpharmacological approach, we then specifically altered the subunit activity through the generation of a dominant-negative mutant. The mutation R299G in γ1, equivalent to R531G in
AMPK-γ2, has been reported to abolish the activity of the AMPK-γ2 subunit without any significant effect on the degree of phosphorylation at Thr172 (36). Figure 4A-J presents an immunoblot obtained with an anti-Myc antibody using HEK cells expressing either the Myc-KCa3.1 channel alone (HIK, lane 1) or in combination with the AMPK-γ1 mutant R299G Myc tagged in COOH-terminus (HIK-R299G, lane 2). The resulting Western blot confirms the expression of the AMPK-γ1-R299G mutant, leading to a band at the expected weight of 38 kDa. In these experiments, β-actin was used as control for total protein loading with HIK cells and HIK cells expressing the negative AMPK-γ1 R299G mutant, respectively (Fig. 4A-2). Figure 4B illustrates an example of inside-out recording of typical cells cotransfected with the AMPK-γ1-R299G mutant (see Fig. 1 for comparison). As seen, the internal addition of AMP under these conditions failed to induce a reduction in KCa3.1 activity, supporting an AMP-dependent regulation of KCa3.1 mediated by AMPK-γ1. These observations are summarized in the frequency histogram presented in Fig. 4C where 5 of the 11 cells studied were found to respond to AMP by an inhibition lower than 20% of the channel activity. This result is clearly at variance with the histogram presented in Fig. 1B where only 1/17 of the cells tested responded to the addition of AMP by an inhibition of 20% or lower of the channel activity. Altogether these observations support the proposal of AMPK activation leading to a decrease in KCa3.1 channel activity.

Interaction between AMPK-γ1 and KCa3.1 in NuLi bronchial monolayers. The evidence gathered so far for protein/protein interactions between KCa3.1 and AMPK-γ1 was obtained in HEK cells overexpressing tagged forms of KCa3.1. To establish if such interactions could be extrapolated to native bronchial cells, KCa3.1/AMPK-γ1 protein/protein interactions were also studied in NuLi bronchial epithelial cell monolayers. Expression of AMPK-γ1 (Fig. 5A, lane 4) and KCa3.1 (Fig. 5C, lane 4) proteins was first demonstrated by Western blot in NuLi cell extracts where bands of 38 and 51 kDa were detected using an anti-AMPK-γ1 or anti-KCa3.1 antibody, respectively. More importantly, the presence of a band at 38 kDa, corresponding to the AMPK-γ1 in Fig. 5A (lane 3) confirmed that KCa3.1 could form an immunoprecipitable complex with AMPK-γ1. Similarly, a band at 51 kDa in Fig. 5C (lane 2) provided clear evidence for KCa3.1 being capable of forming an immunoprecipitable complex with AMPK-γ1. No band was observed when membranes were blotted with the anti-AMPK-γ1 (Fig. 5B, lanes 5, 6, and 7) or anti-KCa3.1 antibodies (Fig. 5D, lanes 3 and 6) in the presence of their respective neutralizing peptides, indicating that the AMPK-γ1 and KCa3.1 signals were specific. Altogether, these results argue for KCa3.1 and AMPK-γ1 interacting in native bronchial cells.

Evidence of KCa3.1 currents in NuLi-polarized monolayers. To determine whether KCa3.1 contributes to the cell ion transport properties, short-circuit measurements were also performed using NuLi cell monolayers. The mean total Isc measured in control conditions was estimated at 29 ± 3 μA/cm² (n = 11), a value similar to that reported previously by Zabner et al. (50). The presence of functional KCa3.1 currents in NuLi monolayers was explored using the KCa3.1 potentiator 1-EBIO (1 mM) applied at the basolateral side. The current record illustrated in Fig. 6A indicates that 1-EBIO induced an additional current of 5.1 ± 0.7 μA/cm² (n = 11) that was completely inhibited by the addition of 5 μM Tram-34, a specific KCa3.1 channel inhibitor. Since it was reported that KCa3.1 channels could be present on both apical and basolateral membranes of human bronchial 16HBE14 cells monolayers (3), the presence of KCa3.1 currents at the apical membrane of NuLi monolayers was also evaluated. Indeed, we observed that 1-EBIO application at the apical side also enhanced Isc currents (Fig. 6, B and C), and this apical 1-EBIO-stimulated current could be reversed by adding Tram-34 on the same side (Fig. 6, B and C). Therefore, these observations provide evidence of functional KCa3.1 channels at both apical and basolateral membranes of NuLi cells.

Impact of AMPK activity on native KCa3.1 currents. To test the impact of AMPK activity on KCa3.1 currents, basal currents (Ibasal, Fig. 5D), 1-EBIO-stimulated Isc (I1-EBIO, Fig. 6E), and 1-EBIO-induced-Tram-34-sensitive currents (ITram-34, Fig. 6F) were compared in nontreated (Ctl) or AICAR-treated (AICAR, 1 mM, 1–2 h) NuLi monolayers. AICAR has been documented as a cell-permeable AMPK activator of which its action is mediated by the internal production of 5-amino-4-

Fig. 3. Membrane colocalization of HA-KCa3.1 channel and AMPK-γ1 in HEK-293 cells. A: immunostaining of HA-KCa3.1 channel performed on permeabilized HEK-293 cells expressing HA-KCa3.1 using a monoclonal anti-HA primary antibody plus an anti-rabbit antibody conjugated to AlexaFluor488 as secondary antibody. B: immunostaining of AMPK-γ1 performed on permeabilized HEK-293 cells expressing HA-KCa3.1 using an anti-AMPK-γ1 primary antibody plus an anti-rabbit antibody conjugated to AlexaFluor594 as secondary antibody. C: overlay of the AlexaFluor488 and AlexaFluor594 staining confirming the localization of AMPK-γ1 at the plasma membrane. Control experiments where no primary antibodies were added did not yield a detectable signal. Single optical sections were obtained by confocal fluorescence microscopy.
DISCUSSION

In this work we provide the first evidence for a regulation of the KCα3.1 channel by the AMP-activated protein kinase. Inside-out patch-clamp measurements showed a decrease in the KCα3.1 activity in response to an increase in AMP concentration, a result supported by two-hybrid screening and pull-down experiments, which confirmed that the COOH-terminal domain of KCα3.1 interacts with the AMP-binding γ1-subunit of AMPK. An AMPK-γ1-based control of KCα3.1 was further suggested by the observations obtained in patch-clamp experiments carried out in cells transfected with the AMPK-γ1 dominant negative R299G mutant where the fraction of cells inhibited by <20% following AMP exposure increased from 5% in control to 50% with the AMPK-γ1-R299G mutant. Finally, we present evidence through coimmunoprecipitation experiments for the formation of a KCα3.1/AMPK-γ1 complex in NuLi cells at endogenous expression levels of AMPK and KCα3.1. Short-circuit current measurements in NuLi cell monolayers confirmed in addition that AMPK activation by AICAR results in a decrease of the KCα3.1-mediated currents, an effect reversed by the AMPK inhibitor Compound-C. Altogether these observations argue for an AMPK-dependent reduction in KCα3.1 channel activity following an increase in AMP-to-ATP ratio.

AMPK as part of a regulatory complex affecting KCα3.1. AMPK has already been documented to modulate the activity of several ion channels, including CFTR (17), the cardiac voltage-gated Na+ channel (29), ENaC (4), and more recently, the Maxi KCα1.1 channel (48). AMPK and CFTR were reported to share an apical distribution in several epithelial tissues (16), and two-hybrid experiments have demonstrated that the COOH-terminal regulatory domain of the AMPK-α1 subunit binds to the COOH-terminal tail of CFTR (18). Evidence was also provided that CFTR can be phosphorylated by AMPK in vitro leading to channel inhibition. Similarly, the α1-subunit of AMPK was found to colocalize at the plasma membrane with the Maxi KCα1.1 channel α-subunit in carotid body-type I cells and to phosphorylate KCα1.1 in vitro (48). These observations are in line with the two-hybrid, pull-down and coimmunoprecipitation results presented in this work, although our data point towards an interaction with KCα3.1 involving the γ1- rather than the α1-subunit. An interaction with AMPK-α1 or AMPK-α2 cannot, however, be entirely ruled out. Such interaction would likely be mediated by AMPK-γ, since AMPK-γ1 and AMPK-α1 or AMPK-α2 are known to participate to the formation of the AMPK complex. It is also clear from our immunohistochemistry experiments that AMPK-γ1 is present a the plasma membrane, in agreement with data demonstrating that the AMPK-β subunit, which is thought to act as a scaffold protein and hold the complex together, is myristoylated (33). Such membrane localization would favor direct KCα3.1 and AMPK-γ1 membrane delimited interactions in accordance with our protein/protein interaction analysis. More importantly, our two-hybrid analysis provided evidence for the amino acids extending from Asp360 to Ala400 to be essential to the interaction between KCα3.1 and the γ1-subunit of AMPK.
As an initial approach to identify a potential phosphorylation site by AMPK on KCa3.1, an analysis was undertaken of the site by AMPK on KCa3.1, an analysis was undertaken of the site. Of AMPK-induced by AMP also reflects cell-to-cell differences in the observed variability in the percentage of current inhibition controlled in our patch-excised experiments, it is likely that the degree of AMPK phosphorylation at Thr172 is not from patch to patch in our inside-out experiments. In addition, the variability in the percentage of current inhibition observed translating the effect of AMPK to various degrees, may account for protein kinases acting simultaneously on KCa3.1, thus modulating the effect of AMPK to various degrees, may account for the variability in the percentage of current inhibition observed from patch to patch in our inside-out experiments. In addition, because the degree of AMPK phosphorylation at Thr172 is not controlled in our patch-excised experiments, it is likely that the observed variability in the percentage of current inhibition induced by AMP also reflects cell-to-cell differences in the initial degree of phosphorylation of the AMPK complex at this site.

As an initial approach to identify a potential phosphorylation site by AMPK on KCa3.1, an analysis was undertaken of the KCa3.1 primary sequence based on the AMPK phosphorylation recognition motif $\phi(X\beta)XXS/TXXX\phi$ where $\phi$ is a hydrophobic and $\beta$ a basic residue (11, 47). This analysis led to the identification of Ser66 as the unique site on KCa3.1 expected to be a substrate to AMPK. As this site is located in the NH$_2$-terminal half of the S2 transmembrane segment, it is unlikely to be phosphorylated by AMPK. We cannot rule out, however, the possibility that nonconventional sequence motifs for phosphorylation sites by AMPK may be present in KCa3.1. Within the limits of our analysis, it would be therefore unlikely for KCa3.1 to constitute per se a substrate to AMPK. AMPK could, however, be attached to KCa3.1 via the $\gamma$-subunit as demonstrated in this work, with the $\alpha$-subunit of AMPK involved in the phosphorylation of an auxiliary protein acting on KCa3.1. One possibility would be CaM, which also contains a potential site of phosphorylation by AMPK. Phosphorylation of CaM by the protein kinase CK2 has been documented to decrease the activity of the small conductance Ca$^{2+}$-activated channel KCa2.2 (5). A similar AMPK-based mechanism could prevail for KCa3.1.

**AMPK regulates KCa3.1 currents in functional epithelia.** NuLi cells, cultured on permeable support at air-liquid interface, have been documented to retain the normal phenotypic qualities of human airway epithelial cells and to form polarized differentiated epithelia that exhibit transepithelial resistance expected for the genotypes (50). Differentiated epithelia that exhibit transepithelial resistance and ion channel physiology expected for the genotypes (50). Short-circuit current measurements carried out on polarized NuLi cell monolayers provide evidence of functional KCa3.1.
channels at both the apical and basolateral membranes. The presence of KCa3.1 in NuLi cells was also confirmed through Western blotting in agreement with our short-circuit current measurements. In our Ussing chamber experiments, KCa3.1 was activated using the KCa3.1 potentiator 1-EBIO (1 mM) to the basolateral side of the monolayer or the apical side of the monolayer caused an increase in $I_{sc}$, which was inhibited by a basolateral application of the KCa3.1 inhibitor Tram-34 (5 μM). This result confirms the presence of KCa3.1 at the basolateral membrane of NuLi cell monolayers. B: similarly, applying 1-EBIO (1 mM) to the apical side of the monolayer caused an increase in $I_{sc}$, which was inhibited by apical application of Tram-34 (5 μM), confirming the presence of KCa3.1 at the apical membrane of NuLi cell monolayers. C: basolateral addition of Tram-34 following apical stimulation of KCa3.1 by 1-EBIO did not result in a significant $I_{sc}$ decrease, confirming the side specificity of the action of Tram-34 seen in B. D–F: impact of AMPK activity on KCa3.1 currents was evaluated by $I_{sc}$ measured in nontreated (Ctl), AICAR-treated (1 mM, 1 h, apical and basolateral side), or AICAR + Compound-C treated (10 μM, 1 h, apical and basolateral side) monolayers. AICAR is used to activate AMPK via the internal production of 5-amino-4-imidazole-carboxamide ribotide (ZMP). Basal short-circuit currents ($I_{basal}$, D; $n = 11$), 1-EBIO-induced currents ($I_{1-EBIO}$, E; $n = 11$), and 1-EBIO-induced, Tram-34 inhibited currents ($I_{Tram-34}$, F; $n = 11$) were then compared in control, AICAR, and AICAR + Compound-C-treated monolayers. The presence of Compound-C succeeded to reverse the effect of AICAR on the KCa3.1-dependent short-circuit currents. These results argue for a control of the KCa3.1 activity by AMPK in NuLi monolayers.

Fig. 6. Impact of AMPK activity on KCa3.1 currents. Short-circuit currents ($I_{sc}$) measured in Ussing chamber on NuLi cell monolayers cultured for 4 to 6 wk, at air-liquid-interface. A: applying the KCa3.1 potentiator 1-EBIO (1 mM) to the basolateral side of the monolayer caused an increase in $I_{sc}$, which was inhibited by a basolateral application of the KCa3.1 inhibitor Tram-34 (5 μM). This result confirms the presence of KCa3.1 at the basolateral membrane of NuLi cell monolayers. B: similarly, applying 1-EBIO (1 mM) to the apical side of the monolayer caused an increase in $I_{sc}$, which was inhibited by apical application of Tram-34 (5 μM), confirming the presence of KCa3.1 at the apical membrane of NuLi cell monolayers. C: basolateral addition of Tram-34 following apical stimulation of KCa3.1 by 1-EBIO did not result in a significant $I_{sc}$ decrease, confirming the side specificity of the action of Tram-34 seen in B. D–F: impact of AMPK activity on KCa3.1 currents was evaluated by $I_{sc}$ measured in nontreated (Ctl), AICAR-treated (1 mM, 1 h, apical and basolateral side), or AICAR + Compound-C treated (10 μM, 1 h, apical and basolateral side) monolayers. AICAR is used to activate AMPK via the internal production of 5-amino-4-imidazole-carboxamide ribotide (ZMP). Basal short-circuit currents ($I_{basal}$, D; $n = 11$), 1-EBIO-induced currents ($I_{1-EBIO}$, E; $n = 11$), and 1-EBIO-induced, Tram-34 inhibited currents ($I_{Tram-34}$, F; $n = 11$) were then compared in control, AICAR, and AICAR + Compound-C-treated monolayers. The presence of Compound-C succeeded to reverse the effect of AICAR on the KCa3.1-dependent short-circuit currents. These results argue for a control of the KCa3.1 activity by AMPK in NuLi monolayers.

channels at both the apical and basolateral membranes. The presence of KCa3.1 in NuLi cells was also confirmed through Western blotting in agreement with our short-circuit current measurements. In our Ussing chamber experiments, KCa3.1 was activated using the KCa3.1 potentiator 1-EBIO and blocked by applying the KCa3.1 inhibitor Tram-34 to either the apical or basolateral side. Because the action of 1-EBIO consists of increasing the channel apparent affinity for Ca$^{2+}$ without changing the cell internal Ca$^{2+}$ level (34), the current inhibition induced by Tram-34 in our experiments is likely to result from a direct block of KCa3.1 and not from a modulation of the cell internal Ca$^{2+}$ concentration as documented elsewhere (38). Furthermore, we found that the application of Tram-34 in absence of 1-EBIO preactivation elicited little, if any, effect on the basal $I_{sc}$ current, thus arguing for an absence of unspecific effect. Finally, we recently detected an 1-EBIO-induced TRAM-34-sensitive current through apically permeabilized NuLi monolayers (in the presence of an apical to basolateral K$^{+}$ gradient), demonstrating the presence of functional KCa3.1 channel at the basolateral membrane of NuLi cells (46). Altogether these observations are in line with previous evidence demonstrating an apical and basolateral distribution of KCa3.1 in wild-type and ΔF508-CFTR expressing human bronchial cell epithelia (3, 12). More importantly, KCa3.1 and AMPK-γ1 interactions could be detected in NuLi cells by coimmunoprecipitation demonstrating that KCa3.1 and AMPK-γ1 can interact under endogenous protein expression levels. These observations thus support the conclusions drawn from systems where KCa3.1 was expressed in HEK cells and argue against KCa3.1/AMPK-γ1 interactions resulting from an overexpression of the KCa3.1 channel.

Our results also demonstrate that activation of AMPK by cell treatment with AICAR caused a significant reduction of the basolateral short-circuit current associated to KCa3.1. The impact of AICAR was abolished by Compound-C. The effect of AICAR was observed in conditions where the AMP-to-ATP ratio remained unchanged because the main effect of AICAR is to cause an accumulation of AICA-ribotide or ZMP inside the cells leading to the activation of AMPK (23). Our observations thus suggest that under metabolic stress conditions, the level of ion and water transepithelial transport could be adjusted as a function of the cell energy status via an effect on KCa3.1. AMPK activation has also been reported to inhibit the cAMP-activated CFTR conductance in Calu-3 cell monolayers (17), through a decrease of the channel open probability. A contribution of CFTR to the effects of AICAR on the 1-EBIO-
induced and Tram-34-sensitive short-circuit currents measured in NuLi cells is unlikely, however, because these experiments were carried out in conditions where CFTR was not activated by cAMP and thus not expected to contribute to the overall transepithelial conductance. Furthermore, a decrease in KCa3.1-mediated currents in NuLi monolayers in response to AICAR treatment is consistent with our inside-out patch-clamp results where internal AMP caused a reduction of the KCa3.1 channel activity at constant ATP and Ca2+ concentrations (23). However, because the action of AICAR requires that the molecule be transformed intracellularly into ZMP, an analog of AMP, the AICAR-based protocol used to induce KCa3.1 inhibition in short-circuit current measurements could not be extended to patch-clamp experiments.

**Physiological implications.** Under normal internal metabolic conditions (ADP/ATP ≈ 0.1), the AMP-to-ATP ratio should approximate 0.01 and be too low to cause AMPK activation (20, 39). The regulation of KCa3.1 by ATP is expected therefore to depend exclusively upon NDPK-B (42) and/or additional Ser/Thr kinases (14). In response to metabolic stressors such as hypoxia or free radicals conditions, however, the ADP-to-ATP ratio can increase up to 0.5 for an AMP-to-ATP ratio of 0.25 (39). Our inside-out patch-clamp results clearly demonstrate that an AMP-to-ATP ratio of 0.2 is sufficient to cause an average 46% decrease in KCa3.1 activity at constant ATP concentration. AMPK activation has been reported to inhibit various diffusive ion-transport pathways, thereby minimizing the dissipation of ionic gradients while conserving cellular ATP by limiting ATP consumption via ATP-dependent transport processes. Because the vectorial transport of ions in Cl−-secreting epithelial cells is strongly modulated by the K+ conductance at the basolateral membrane, our results show that KCa3.1 inhibition through AMPK activation contribute to reinforce the downregulation of ion transport in these cells. The role of KCa3.1 might be, in this regard, rather crucial as one of the expected effects of metabolic stressors is to cause an increase of the intracellular Ca2+ concentration (1), thus favoring a strong KCa3.1 activation. A downregulation of KCa3.1 by AMPK transport would under these conditions preserve the intracellular ionic environment and defend the ability of the cell to generate ATP in the face of metabolic stress (4). Because AMPK acts as a metabolic sensor, AMPK activation might also determine cell survival in pathological conditions including cystic fibrosis (CF). In line with this proposal is the observation of an increased AMPK protein expression in airway cells from CF patients relative to cells from non-CF patients (15). A concomitant downregulation of KCa3.1 and ENaC by AMPK is therefore susceptible to minimize the deleterious effect of a Na+ hyperabsorption through ENaC in CF conditions. Overall our results are in agreement with a model whereby the response of Cl−-secreting epithelial cells to a metabolic stress such as the one prevailing in CF conditions due to chronic infection or inflammation involves the common inhibition of both CFTR and KCa3.1 while decreasing the number of functional ENaC channels at the apical membrane (7, 15, 17).

In conclusion, AMPK is now considered as a cellular “fuel-gauge” involved in the regulation of energy metabolism. Our results supporting a regulation of KCa3.1 gating by AMPK point towards a global modification of the ion transport properties in Cl−-secreting epithelia not exclusively mediated by CFTR and ENaC, but that includes a contribution of KCa3.1 as well.

**ACKNOWLEDGMENTS.** We acknowledge the help of Julie Verner for expert cell preparation and M. Michel Lauzon for technical assistance for confocal imaging. The authors also thank Ariane Longpré-Lauzon for critical reading of the manuscript.

**GRANTS.** This work was supported by grants from the Canadian Cystic Fibrosis Foundation (to R. Sauvé and E. Brochiero) and from the Institutes of Health Research (MOP 7769 to R. Sauvé).

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