

# Functional Apical Large Conductance, Ca<sup>2+</sup>-activated, and Voltage-dependent K<sup>+</sup> Channels Are Required for Maintenance of Airway Surface Liquid Volume\*

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Large conductance, Ca<sup>2+</sup>-activated, and voltage-dependent K<sup>+</sup> (BK) channels control a variety of physiological processes in nervous, muscular, and renal epithelial tissues. In bronchial airway epithelia, extracellular ATP-mediated, apical increases in intracellular Ca<sup>2+</sup> are important signals for ion movement through the apical membrane and regulation of water secretion. Although other, mainly basolaterally expressed K<sup>+</sup> channels are recognized as modulators of ion transport in airway epithelial cells, the role of BK in this process, especially as a regulator of airway surface liquid volume, has not been examined. Using patch clamp and Ussing chamber approaches, this study reveals that BK channels are present and functional at the apical membrane of airway epithelial cells. BK channels open in response to ATP stimulation at the apical membrane and allow K<sup>+</sup> flux to the airway surface liquid, whereas no functional BK channels were found basolaterally. Ion transport modeling supports the notion that apically expressed BK channels are part of an apical loop current, favoring apical Cl<sup>-</sup> efflux. Importantly, apical BK channels were found to be critical for the maintenance of adequate airway surface liquid volume because continuous inhibition of BK channels or knockdown of *KCNMA1*, the gene coding for the BK  $\alpha$  subunit (*KCNMA1*), lead to airway surface dehydration and thus periciliary fluid height collapse revealed by low ciliary beat frequency that could be fully rescued by addition of apical fluid. Thus, apical BK channels play an important, previously unrecognized role in maintaining adequate airway surface hydration.

Large conductance, voltage- and Ca<sup>2+</sup>-activated K<sup>+</sup> (BK)<sup>3</sup> channels control a variety of physiological processes in different tissues. The BK pore-forming structure is a homotetramer of the  $\alpha$  subunit (*KCNMA1*, Slo1, KCa1.1), which is encoded by a single gene (*Slo1* gene, named *KCNMA1*). Four  $\beta$  regulatory

subunits (*KCNMB1–4*), encoded by the genes *KCNMB1–B4*, modulate the kinetics and the calcium and voltage dependence of BK channels, thereby contributing to the functional versatility of BK in different tissues (1–6).

Ion transport plays an important role in maintaining adequate water supply for the apical airway surface, which is critical for mucus hydration and ciliary beating because the periciliary fluid level has to be maintained for cilia to be effective. Both mucus hydration and ciliary beating are critical components of mucociliary function. The importance of ion transport for adequate airway surface liquid (ASL) volume is illustrated by multiple airway diseases where decreased Cl<sup>-</sup> secretion and increased Na<sup>+</sup> absorption cause airway surface dehydration and thereby mucociliary dysfunction. A prime example is cystic fibrosis, a disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (7, 8). However, other channels contribute directly to or maintain the electrochemical gradient necessary for apical Cl<sup>-</sup> secretion. For example, calcium-activated chloride channels, recently identified as TMEM16 (9–11), secrete Cl<sup>-</sup> and are important for airway hydration (12). In addition, basolateral K<sup>+</sup> channels contribute to apical Cl<sup>-</sup> transport by maintaining the electrochemical gradient required for Cl<sup>-</sup> movement (13–17). Mall *et al.* (17) found that UTP-induced Cl<sup>-</sup> currents in human nasal tissue were dependent on both clotrimazole-sensitive, calcium-activated K<sup>+</sup> channels (SK4, KCa3.1, gene *KCNN4*) and clofilium-sensitive voltage-activated K<sup>+</sup> channels (hKvLQT1, gene *KCNQ1*). Bernard *et al.* (18) also found that SK4 channels contribute to calcium-dependent chloride secretion in the human bronchial cell line 16HBE14o-

Physiological ATP release onto apical surfaces of airway epithelial cells plays an important role in regulating water balance (19–21) and thereby mucociliary transport (22). Apical ATP is well known to increase [Ca<sup>2+</sup>]<sub>i</sub> via P<sub>2</sub>Y<sub>2</sub> receptors. Because BK channels are sensitive to intracellular Ca<sup>2+</sup>, we hypothesized that these channels may be involved in ion transport responses to apically released ATP in human bronchial epithelia, which would make these channels important for the regulation of ASL volume in normal human bronchial epithelial (NHBE) cells.

Using electrophysiological techniques (patch clamp and Ussing chamber) and RNA level modifications, we identified functional BK channels at the apical but not basolateral membrane of fully differentiated NHBE cells. These apical BK channels play a key role in ion transport in response to apically

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<sup>3</sup> The abbreviations used are: BK, large conductance, Ca<sup>2+</sup>-activated, and voltage-dependent K<sup>+</sup>; NHBE, normal human bronchial epithelial; ALI, air-liquid interface; qPCR, quantitative PCR;  $\Omega$ , ohm; TES, *N*-tris-(hydroxymethyl) methyl-2-amino-ethane-sulfonic acid; CBF, ciliary beat frequency.

**TABLE 1**  
Taqman assay for qPCR of BK  $\alpha$  and  $\beta$  subunits

Assay ID	mRNA	Gene	Gene name (alias)
Hs00266938_m1	NM_001014797, NM_002247, NM_001161352.1, NM_001161353.1	KCNMA1	Potassium large conductance calcium-activated channel, subfamily M, $\alpha$ member 1 (MaxiK, BK <sub>Ca</sub> , KCa1.1)
Hs00188073_m1	NM_004137.2	KCNMB1	Potassium large conductance calcium-activated channel, subfamily M $\beta$ member 1
Hs00175772_m1	NM_181361.1, NM_005832.3	KCNMB2	Potassium large conductance calcium-activated channel, subfamily M $\beta$ member 2
Hs00205246_m1	NM_171828.1, NM_171829.1, NM_171830.1, NM_014407.3	KCNMB3	Potassium large conductance calcium-activated channel, subfamily M $\beta$ member 3
Hs00205704_m1	NM_014505.4	KCNMB4	Potassium large conductance calcium-activated channel, subfamily M, $\beta$ member 4
Hs99999905_m1	NM_002046.3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (G3PD, GAPD)

released ATP as loss of their activity results in airway surface dehydration. Thus, apical BK channels physiologically modulate ASL volume, which is critical for mucus hydration and ciliary beating.

## EXPERIMENTAL PROCEDURES

**Chemicals and Solutions**—All media and Hank's balanced salt solution were purchased from Invitrogen. Unless stated otherwise, all other materials were obtained from Sigma Aldrich.

**Air-liquid Interface (ALI) Cell Culture**—Normal human airways were obtained from organ donors whose lungs were rejected for transplant. Institutional Review Board-approved consent for research with these tissues was obtained by the Life Alliance Organ Recovery Agency of the University of Miami and conformed to the declaration of Helsinki. Airway epithelial cells were isolated and used for experiments or dedifferentiated through expansion. Passage 1 cells were redifferentiated at an air-liquid interface (ALI) on collagen-coated 24-mm T-clear or 12 mm Snapwell filters (Costar Corning) for ~3–4 weeks (at which time cultures were ciliated and secreted mucus) as described previously (23–26). All experiments were repeated  $\geq 3$  times from at least two different lungs.

**RNA Expression**—Total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems) with the TaqMan Gene Expression Assays (Applied Biosystems) described in Table 1 according to the manufacturer's instructions. Thermal cycling was carried out using an ICycler IQ apparatus (Bio-Rad). Each sample was analyzed in triplicate. The difference in the threshold cycle between the targeted gene and the housekeeping gene GAPDH ( $\Delta Ct$ ) was used as an estimation of the relative level of expression. qPCR was also used to calculate relative gene expression after knockdown experiments (see below).

**Western Blot**—ALI cultured cells were solubilized in SDS, and proteins were separated by PAGE and blotted onto Immobilon-P membranes (Millipore, Billerica, MA). For detection of the BK  $\alpha$  subunit, a primary rabbit anti-human BK ( $\alpha$  subunit) antibody (Sigma-Aldrich catalog no. P4872) was used, and a secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was used for chemiluminescence. To normalize for protein loading, membranes were reprobbed with rabbit anti- $\beta$ -ac-

tin (1:100) (Sigma-Aldrich). Band intensities were quantified in the linear range using a Bio-Rad Chemidoc XRS.

**Single Channel Current Experiments**—Freshly isolated cells or fully differentiated NHBE cells after trypsinization were plated on collagen-coated 35-mm Petri dishes in ALI medium and kept from 20 to 24 h for recovery. Patch pipettes of 0.2  $\mu$ m in diameter were pulled from melting point tubes (product no. 9530-1; Corning Glass Works) and had a resistance between 10–15 M $\Omega$ . No series resistance compensation was used, but the error due to uncompensated series resistance was never  $> 5$  mV. Single-channel K<sup>+</sup> currents were recorded from excised patches of membranes from ciliated cells (inside-out or outside-out) with the pipette and bath solutions both containing 150 mM KCl, 20 mM sucrose, 10–100  $\mu$ M CaCl<sub>2</sub>, 10 mM *N*-tris-(hydroxymethyl) methyl-2-amino-ethane-sulfonic acid (TES), adjusted to pH 7.0. Experiments were performed at room temperature (21–23 °C), and data were collected with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered with a low-pass Bessel filter at 5 kHz, and sampled at 200 kHz with a Digidata 1322A, driving a 16-bit analog interface. The acquisition and basic analysis of the data were performed with pClamp software (version 9.2; Axon Instruments, Inc.).

**Macroscopic Current Experiments**—Intact, fully differentiated NHBE cells cultured at the ALI were recorded for macroscopic currents in cell-attached, outside-out, and excised inside-out patches, using the same glass patch pipettes with diameter 1–3  $\mu$ m and 1–3 M $\Omega$  resistance. Intracellular solutions contained the following: 110 mM potassium methanesulfonate, 10 mM HEPES, 2 mM CaCl<sub>2</sub>, pH 7.2. Extracellular solution contained ND-96: 96 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 500 mg/ml gentamycin, 5 mM HEPES, pH 7.2. For macroscopic current relaxation experiments, the membrane was held at –60 mV. Each experiment consisted of a time at the holding potential, followed by a depolarization to test voltages between –60 mV to 100 mV, in 20-mV increments, separated by 10 s. The tail currents were at –60 mV. The analog signal was filtered before digitization with an eight-pole low-pass Bessel filter with a cutoff frequency of 1/5 of the conversion frequency.

**Ussing Chamber Experiments**—Fully differentiated NHBE cells grown on Snapwell filters (1.13 cm<sup>2</sup>) were mounted in Ussing chambers (EasyMount Chamber) connected to a VCC MC6 voltage clamp unit (both from Physiologic Instruments, San Diego, CA). Solutions were maintained at 37 °C by heated water jackets. To monitor short circuit current ( $I_{sc}$ ), transepithelial membrane potential was clamped to 0 mV. Only cultures

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**TABLE 2**

**Hairpin sequences used to knockdown KCNMA1 in NHBE cells**

Mature sense and antisense sequences are in boldface letters. TRC, The RNAi Consortium Library Database.

TRC no.	Targeted RNA	Hairpin sequence
SHC002	MISSION nontarget shRNA Control Vector	CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGGCTCTTCATCTTGTGTTT
TRCN0000000212	NM_001014797 NM_002247	CCGGGTCAAGATAGAGTCAGCAGATCTCGAGATCTGCTGACTCTATCTTGACTTTT

with  $>200 \Omega \text{ cm}^{-2}$  were used for the experiments. Signals were digitized and recorded with DAQplot software (VVI Software, College Station, PA) via a LabJack A/D converter (LabJack Corp., Lakewood, CO). All experiments were conducted with culture and date matched filters.

Apical  $\text{K}^+$  currents were measured 30 min after permeabilization according a protocol published by Namkung *et al.* (27) (20  $\mu\text{M}$  amphotericin B, 10  $\mu\text{M}$  valinomycin, 10  $\mu\text{M}$  nigericin), in the presence of apical 10  $\mu\text{M}$  amiloride. A  $\text{K}^+$  gradient was created with one solution containing 145 mM  $\text{K}^+$  gluconate, 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{Ca}^{2+}$  gluconate, 10 mM glucose, 10 mM HEPES, pH 7.4, and a another solution in which 140 mM  $\text{K}^+$  gluconate was replaced by  $\text{Na}^+$  gluconate. Resistance measurements showed complete permeabilization of the basolateral membrane within 30 min of addition of amphotericin B, valinomycin, and nigericin, indicated by a decrease of  $16 \pm 3\%$  in total resistance ( $n = 8$ ), which is within a reasonable range of the theoretical drop of 17% in total resistance when fully permeabilizing the basolateral membrane calculated from the measured apical and basolateral membrane (in series) as well as paracellular resistances (in parallel) reported by Willumsen *et al.* (28).

**KCNMA1 Knockdown**—Lentiviral pLKO plasmids were used as described (24, 29). From 14 to 21 days at the ALI, when beating cilia became apparent, KCNMA1 knockdown cells and nontargeting lentivirus infected cells (Table 2) were used for qPCR, Western blots, Ussing chamber experiments, and ciliary beat measurements (CBF).

**Computer Simulations**—Computer simulations were done using the model of Cook and Young (30) with modifications and in-house software. The model contains the following assumptions (30): (a) the secreted fluid was assumed to be isotonic, *i.e.*  $[\text{Cl}]_l = [\text{Na}]_l + [\text{K}]_l = [\text{Na}]_i + [\text{K}]_i = [\text{Cl}]_i$  where  $l$  is luminal and  $i$  is interstitial; (b) the cytosolic composition was assumed to not vary with the secretory rate; (c) the ion transport across the epithelia was assumed to be at steady state; (d) the net ion fluxes into the luminal compartment determine the composition of the luminal fluid, *i.e.*  $[\text{Na}]_l = [\text{Cl}]_i (I_i^{\text{Na}} + I_a^{\text{Na}}) / I_a^{\text{Cl}}$ ; (e) the pump current ( $I_p$ ) is one sixth of the apical  $\text{Cl}^-$  current; and (f) the resistances of the tight junctions are inversely proportional to the mean of the concentrations of  $\text{Na}^+$  (or  $\text{K}^+$ ) in the luminal and interstitial solutions, *e.g.*  $R_j^{\text{Na}} = (2 \times 155) p_j^{\text{Na}} / ([\text{Na}]_l + [\text{Na}]_i)$ . The parameters used in the simulations are shown in Table 3.

**$[\text{Ca}^{2+}]_i$  Estimates in NHBE Cells**—NHBE cells were loaded with 5  $\mu\text{M}$  fura-2/AM. Ratiometric fura-2 fluorescence in single cells was measured as described (31).

**Measurement of CBF as a Surrogate for ASL Volume**—Adequate ASL volume maintains an appropriate periciliary fluid height that allows normal ciliary activity. Thus, conditions that

**TABLE 3**

**Parameters used in the computer simulations**

Parameters as in Cook and Young (30) based on values from dog tracheal mucosa (62, 63).  $R_a^{\text{K}}$  (min) is the apical  $\text{K}^+$  resistance at the maximum apical  $\text{K}^+$  conductance used in the simulations (Fig. 5). We used two different values for the junctional resistance: 300 or 600  $\Omega \text{ cm}^2$ , which are close to the estimated paracellular resistance of 412  $\Omega \text{ cm}^2$  in normal nasal epithelia (28).  $R_a^{\text{Na}}$  was chosen to yield an amiloride-sensitive  $I_{sc}$  that was 35% of the total  $I_{sc}$ , as found experimentally with our cells (data not shown).

$P_i^{\text{Na}}$	300 or 600 $\Omega \text{ cm}^2$
$P_i^{\text{K}}$	300 or 600 $\Omega \text{ cm}^2$
$R_a^{\text{Cl}}$	274 $\Omega \text{ cm}^2$
$1/(1/R_{bl}^{\text{K}} + 1/R_a^{\text{K}}(\text{min}))$	154 $\Omega \text{ cm}^2$
$R_a^{\text{Na}}$	8000 $\Omega \text{ cm}^2$
$[\text{Cl}]_l$	160 mmol/liter
$[\text{Na}]_l$	155 mmol/liter
$[\text{K}]_l$	5 mmol/liter
$[\text{Cl}]_i$	54 mmol/liter
$[\text{K}]_i$	155 mmol/liter
$[\text{Na}]_i$	5 mmol/liter

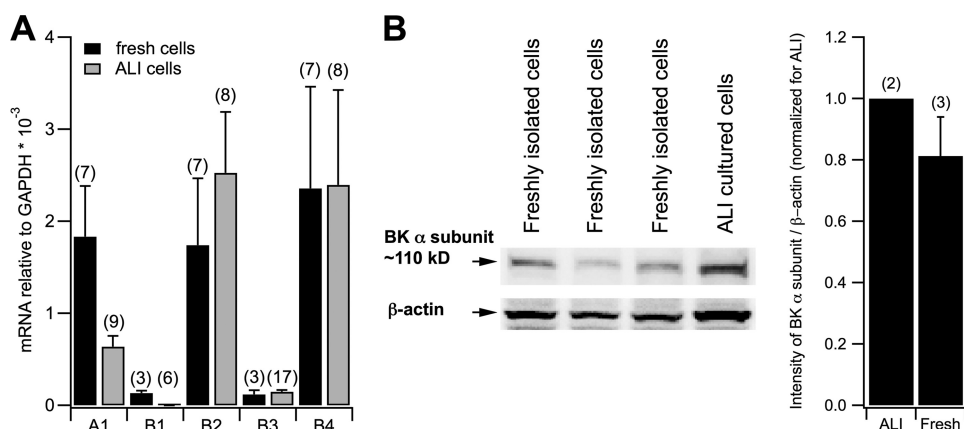
are associated with a CBF reduction that is fully rescued upon fluid addition to the apical compartment indicate ASL volume depletion. For these experiments, regular washing of the apical surface with Dulbecco's PBS was not performed for 3–4 days. Cells were imaged on a Zeiss Axiovert 200, equipped with phase contrast optics. CBF was measured as described (32). All the measurements were done within a 0.5-cm radius from the center of each 24-mm filter (no influence of possible water menisci at the edges of the cultures).

**Statistics**—Results were analyzed by a Student's  $t$  test for comparing two groups. One-way analysis of variance followed by a Tukey honestly significant difference test for mean comparison was used for more than two groups.  $p < 0.05$  was accepted as significant. Other statistics are indicated in the figures. Data are presented as mean  $\pm$  S.E.

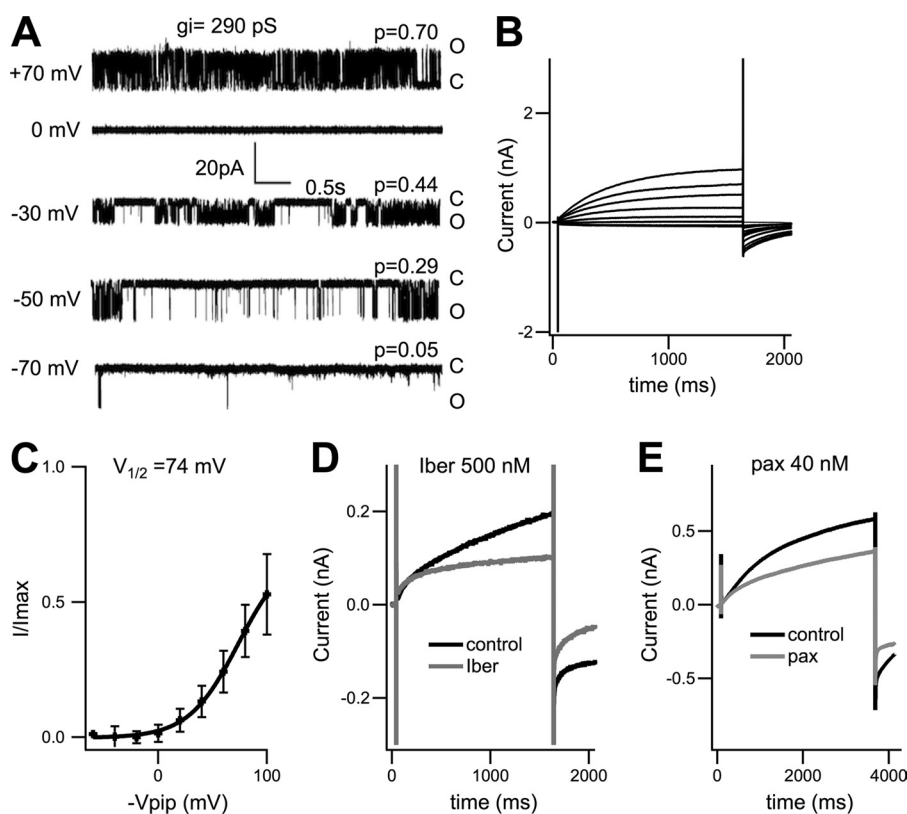
## RESULTS

**BK Channels Are Expressed in Freshly Isolated and ALI-cultured NHBE Cells**—qPCR using RNA isolated from freshly isolated airway epithelial cells and NHBE cells redifferentiated at the ALI revealed mRNA expression of the  $\alpha$  subunit and the  $\beta 2$  and  $\beta 4$  regulatory subunits, whereas the  $\beta 3$  subunit expression was low, and the  $\beta 1$  mRNA expression was low to undetectable (Fig. 1A). Both  $\beta 2$  (1, 33, 34) and three of the four splice variants of the  $\beta 3$  subunit (1, 33–36) inactivate BK channel currents. The  $\beta 4$  subunit changes the kinetics of the channel (1) and its calcium dependence (37). Therefore, the kinetics of BK activity is probably modulated by the presence of several regulatory subunits in NHBE cells. By Western blot, an immunoreactive band of the predicted size for KCNMA1 at  $\sim 110$  kDa was detected at approximately the same amount in both NHBE cells cultured at the ALI and in freshly isolated cells (Fig. 1B).

**BK Channels Are Functional at Apical Membrane**—To prove that BK channels were functional in our cells, single channel



**FIGURE 1. BK channels are expressed in freshly isolated airway epithelial cells and NHBE cells redifferentiated at the ALI.** *A*, qPCR analysis of the expression of *KCNMA1* (mRNA) and *KCNMB1-4* (mRNA) in freshly isolated airway epithelial cells (*black bars*) and NHBE cells fully redifferentiated at the ALI (*gray bars*). Values shown are mean ± S.E. of relative expression levels compared with GAPDH (mRNA) (each *n* shows the number of qPCRs from >3 different lungs, indicated in *parentheses* above the corresponding *bar*). *B*, Western blot for BK α subunit. Lysates from fully differentiated NHBE cells (20 μg total protein in each lane) were electrophoresed, transferred, and probed with a polyclonal antibody to the α subunit of the BK channel (Sigma-Aldrich, P4872). Freshly isolated cells express similar amounts of the BK α subunit protein when loading was normalized with β-actin.

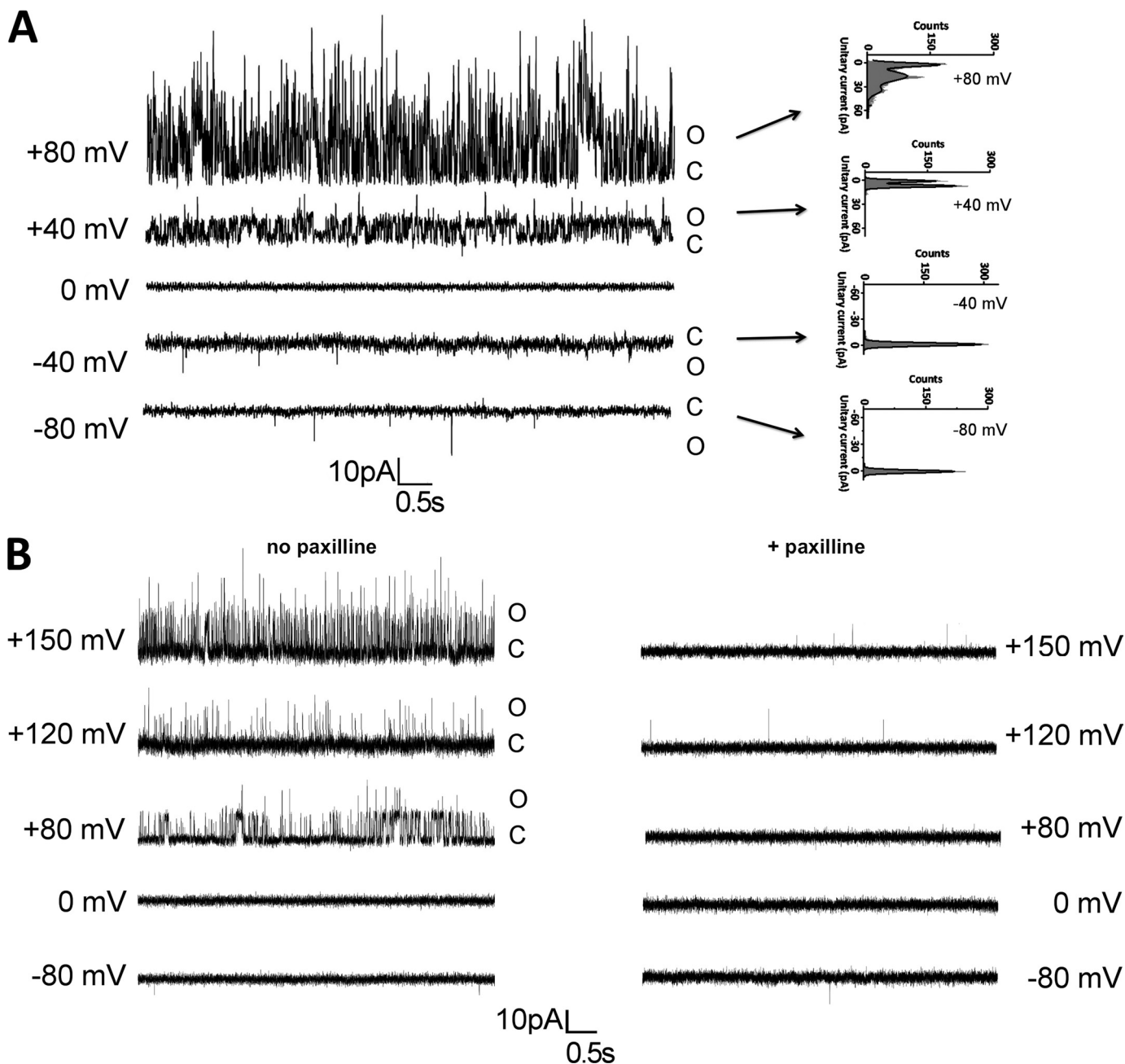


**FIGURE 2. BK channels are functional in NHBE cells cultured at the ALI.** *A*, family of single channel BK currents. The traces were elicited at  $-70$ ,  $-50$ ,  $-30$ ,  $0$ , and  $+70$  mV in trypsinized NHBE cells using the inside-out patch configuration. The  $\text{Ca}^{2+}$  concentration in both the pipette and bath was  $100 \mu\text{M}$ . The single conductance calculated was  $\sim 290$  picosiemens. Note that the open probability increases at more positive voltages. *C*, closed; *O*, open. *B*, functional  $\text{K}^+$  channels in intact (nontypsinized) NHBE cells cultured at the ALI. Representative macroscopic currents are shown, which were measured in the cell-attached patch configuration. The holding potential was  $-60$  mV (*i.e.*  $V_{\text{pipette}} = +60$  mV), and the membrane was pulsed to voltages between  $-60$  to  $+100$  mV, in increments of  $-20$  mV following a step to  $-60$  mV. *C*, voltage-dependent activation curve obtained from macroscopic currents. Each point is an average of three to four patches obtained from the apical side of intact NHBE cells. The curve was fit to a simple Boltzmann equation, with a  $V_{1/2} = 74$  mV. *D*, effect of iberiotoxin (*iber*;  $500$  nM) on currents in response to a step to  $+40$  mV from an outside-out macropatch obtained from the apical surface of an intact, fully differentiated ALI culture of NHBE cells. *E*, effect of  $40$  nM paxilline (*pax*) on currents in response to a step to  $+40$  mV from a cell-attached macropatch obtained from the apical surface of an intact, fully differentiated ALI culture of NHBE cells.

currents were measured in inside-out patches of trypsinized NHBE cells (Fig. 2A). At  $0$  mV, currents were close to  $0$ , suggesting that the reversal potential matched the expected potential for  $\text{K}^+$  currents in symmetrical  $\text{K}^+$  solutions. When screened from  $-70$  to  $+70$  mV, all currents were found to have

a single conductance of  $\sim 290$  picosiemens. The voltage dependence of the currents and, in particular, their high conductance was in agreement with the properties expected for BK channels. We also recorded similar, BK channel-like currents from freshly isolated airway epithelial cells (see Fig. 3 below).

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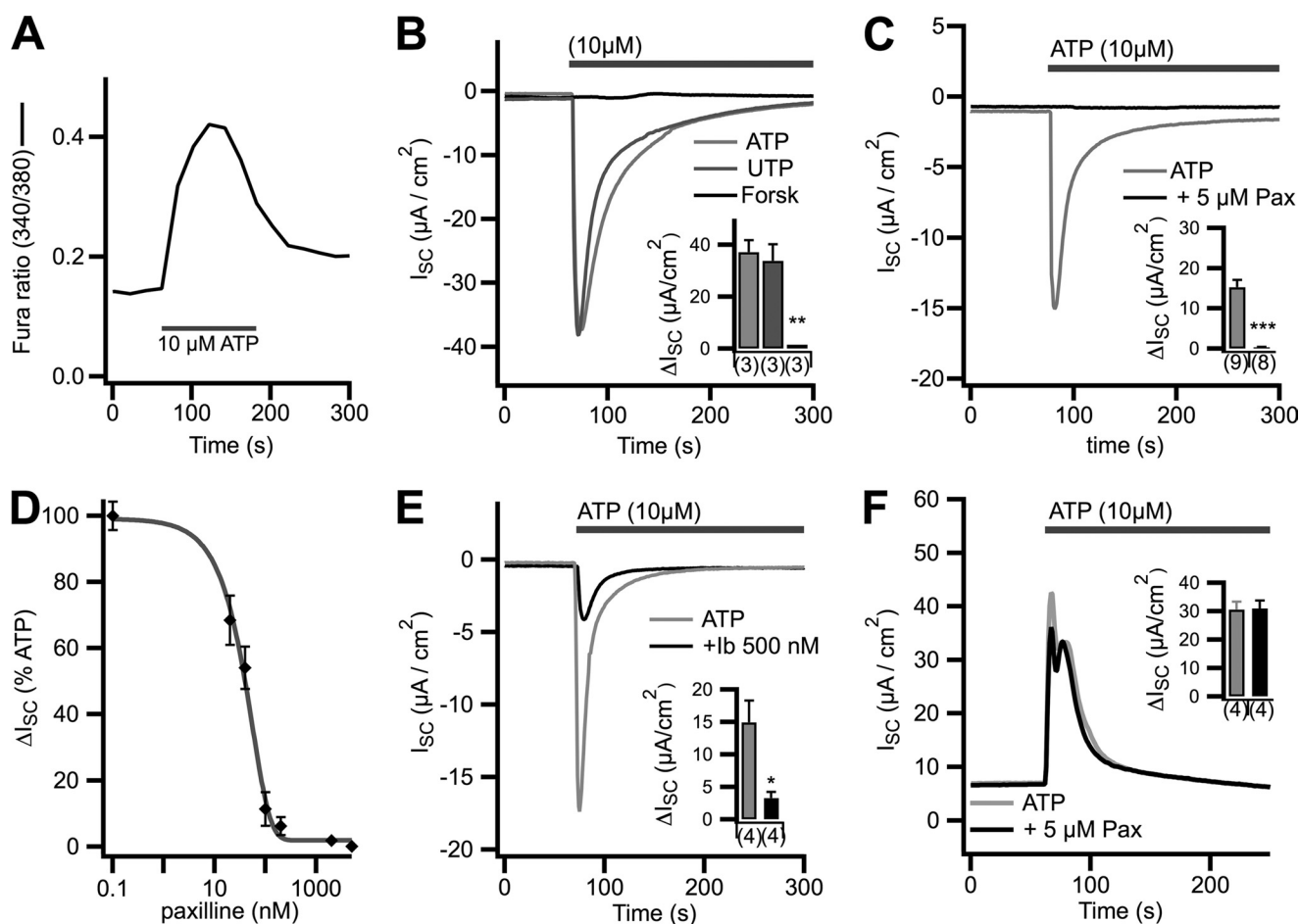
**FIGURE 3. BK channels are functional in freshly isolated airway epithelial cells.** *A*, family of single channel BK currents from a freshly isolated ciliated cell (not redifferentiated). The traces were elicited at  $-80$ ,  $-40$ ,  $0$ ,  $+40$ , and  $+80$  mV using the inside-out patch configuration with a  $\text{Ca}^{2+}$  concentration in both pipette and bath of  $100 \mu\text{M}$ . At high positive voltage, there are several channels present in the patch. The single conductance calculated at  $275$  picosiemens. *B*, family of single channel BK currents from a freshly isolated ciliated cell (not redifferentiated) in the outside out configuration before (*left*) and after paxilline treatment ( $50 \mu\text{M}$ ; *right*) in the presence of a  $\text{Ca}^{2+}$  concentration in both pipette and bath of  $10 \mu\text{M}$ . Note that paxilline almost completely eliminates channel openings and that the channels are sensitive to  $\text{Ca}^{2+}$  (at lower  $\text{Ca}^{2+}$  concentrations here than in *A*, more voltage is needed to open the channel). *C*, closed; *O*, open.

To eliminate potential artifacts derived from trypsinization, we performed patch clamp recordings of macroscopic currents of intact, *i.e.* not permeabilized, but fully differentiated NHBE cells grown at the ALI. Recordings in the cell-attached configuration show voltage-dependent currents (Fig. 2*B*) with a  $V_{1/2}$  consistent with expected values for BK channels (*e.g.* Refs. 38 and 39) for resting low intracellular  $\text{Ca}^{2+}$  levels under these conditions (Fig. 2*C*).

To test whether this current was due to BK channels, the effect of BK blockers was examined. Iberitoxin ( $500 \text{ nM}$ ) reduced the total currents by  $50\%$  at  $1.4 \text{ s}$  (Fig. 2*D*) when applied

in the outside-out patch configuration. Paxilline ( $40 \text{ nM}$ ), an indole diterpene, cell-permeable BK blocker ( $40$ ), applied apically in the cell-attached configuration elicited a current reduction of  $\sim 40\%$  at  $1.4 \text{ s}$  (Fig. 2*E*), suggesting apical expression of functional BK channels. BK currents with similar properties were also recorded from freshly isolated ciliated cells with characteristics similar to the cells redifferentiated at the ALI (Fig. 3, *A* and *B*).

These experiments constitute the first evidence of BK channel activity in freshly isolated ciliated and fully redifferentiated, polarized human airway epithelia with tight junctions intact.



**FIGURE 4. Nucleotide-stimulated BK channel activity can be recorded apically but not basolaterally in fully differentiated ALI cultures of NHBE cells.** A, recording of fura-2 fluorescence from a single cell. As expected, 10  $\mu\text{M}$  ATP transiently increases  $[\text{Ca}^{2+}]_i$  (31). The duration of ATP exposure in this example is 2 min; however, longer exposures result in similar traces (see Fig. 2 in Ref. 31). B–E, Ussing chamber experiments in basolaterally permeabilized cells exposed to a basolateral to apical  $\text{K}^+$  gradient in the presence of 10  $\mu\text{M}$  amiloride (to block  $\text{Na}^+$  current in the apical membrane). All compounds were added apically. B, a representative experiment (main panel) and quantification of the responses (inset) of outward short circuit currents ( $I_{sc}$ ) in response to 4 min of 10  $\mu\text{M}$  ATP, 10  $\mu\text{M}$  UTP, or 10  $\mu\text{M}$  forskolin (Forsk). Note that negative  $I_{sc}$  corresponds to outward  $\text{K}^+$  flux in these Ussing chamber experiments. The bars of the insets are reported as mean  $\pm$  S.E. of the number of cultures from three different lungs indicated in the parentheses below each bar. C, effect of 5  $\mu\text{M}$  paxilline, a BK channel inhibitor (preincubation for 3–5 min) on ATP-induced  $I_{sc}$  changes. D, dose-dependent inhibition of 10  $\mu\text{M}$  ATP-induced  $I_{sc}$  changes by paxilline. Nonlinear regression fit for log inhibitor versus normalized response gave best fit values of  $\log \text{IC}_{50} = 1.557 \pm 0.03856$  nm and  $\text{IC}_{50} = 36.5$  nm ( $r^2 = 0.9650$ ,  $n = 22$ ). E, effect of iberiotoxin (Ib, 500 nM, 30-min preincubation) on ATP-induced  $I_{sc}$  changes. F, Ussing chamber experiments in apically permeabilized cells exposed to an apical to basolateral  $\text{K}^+$  gradient. All compounds were added basolaterally. Although 10  $\mu\text{M}$  ATP induced a change in  $I_{sc}$  (basolateral  $\text{K}^+$  secretion), this change was not inhibited by the BK channel blocker paxilline, indicating that BK channels are active only at the apical membrane. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$ .

Thus, we explored next whether only apical or also basolateral BK activity can be identified.

**ATP Activates Apical BK Channels in NHBE Cells, whereas No BK-dependent Current Can Be Recorded Basolaterally**—Apical ATP and UTP are well known to increase  $[\text{Ca}^{2+}]_i$  via  $\text{P}_2\text{Y}_2$  receptors, an important physiological signal for airway epithelial homeostasis. To show that apical ATP elicits a transient  $[\text{Ca}^{2+}]_i$  increase in these NHBE cells, fura-2 fluorescence was recorded from single cells upon 10  $\mu\text{M}$  ATP exposure. A typical, transient  $[\text{Ca}^{2+}]_i$  increase is shown in Fig. 4A. To test for  $\text{Ca}^{2+}$ -stimulated, apical, and BK channel-dependent  $\text{K}^+$  currents, fully differentiated, basolaterally permeabilized NHBE cells were mounted in Ussing chambers for  $I_{sc}$  measurements ( $V_c = 0$  mV) using a basolateral to apical  $\text{K}^+$  gradient to assess apical  $\text{K}^+$  efflux (Fig. 4, B–E). In basolaterally permeabilized ALI cells, both 10  $\mu\text{M}$  ATP and UTP induced a strong change in  $I_{sc}$ , consistent with apical  $\text{K}^+$  efflux (Fig. 4B). On the

other hand, forskolin, a direct activator of transmembrane adenylyl cyclases to increase cyclic AMP, did not change  $I_{sc}$  significantly (Fig. 4B). This differential effect of nucleotides and forskolin on  $I_{sc}$  suggests that the  $I_{sc}$  changes could be linked to the opening of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels, possibly BK channels, and not channels known to be activated by forskolin such as KCNQ1 (27). Indeed, the ATP-stimulated  $I_{sc}$  increase was blocked by apical application of paxilline in a dose-dependent manner, with 36.5 nM paxilline causing half-maximal inhibition (Fig. 4, C and D). These values compare well with the half maximal inhibition concentration of paxilline found in macroscopic currents during patch clamp experiments (40 nM; Fig. 2E). In fact, 5  $\mu\text{M}$  paxilline completely inhibited the ATP-induced increases in  $I_{sc}$  (Fig. 4C). Apical application of 5  $\mu\text{M}$  paxilline did not affect baseline currents in these basolaterally permeabilized NHBE cells, which is in agreement with patch clamp experiments showing  $I/I_{\text{max}}$

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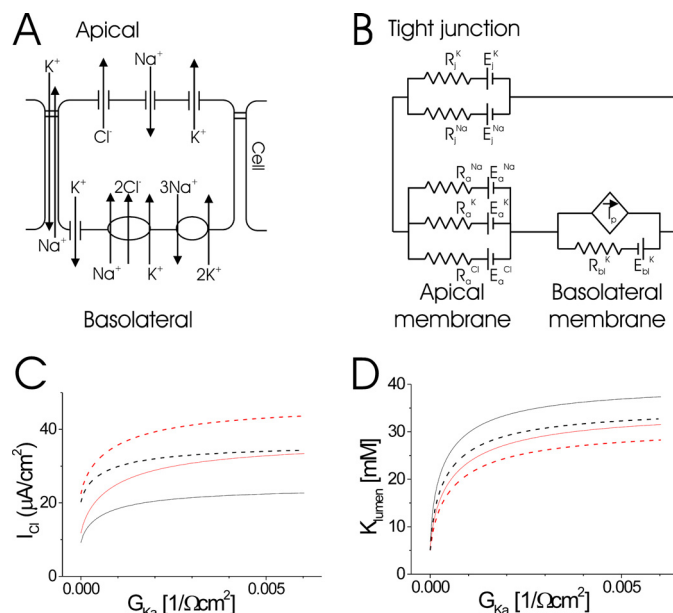
close to 0 (due to low basal cytosolic  $\text{Ca}^{2+}$  concentrations) at 0 mV in intact cultures. In addition, 500 nM iberiotoxin applied apically 30 min before measurements blocked  $\sim 80\%$  of the  $I_{sc}$  response to  $10 \mu\text{M}$  ATP (Fig. 4E).

To test for basolateral, BK channel-dependent  $\text{K}^+$  currents, fully differentiated, apically permeabilized NHBE cells were mounted in Ussing chambers for  $I_{sc}$  measurements (at  $V_c = 0$  mV) using an apical to basolateral  $\text{K}^+$  gradient to assess basolateral  $\text{K}^+$  efflux (Fig. 4F). The outward (basolateral) current elicited by  $10 \mu\text{M}$  ATP at the basolateral membrane was not significantly influenced by  $5 \mu\text{M}$  paxilline, indicating that BK channels are solely active in the apical membrane in these conditions.

**Simulations Support Hypothesis That Apical  $\text{K}^+$  Currents Increase Apical  $\text{Cl}^-$  Efflux**—It is known that basolateral  $\text{K}^+$  efflux accompanies apical  $\text{Cl}^-$  secretion, resulting in a transepithelial electrical potential that in turns drives paracellular  $\text{Na}^+$  transport, which finally generates the osmotic gradient that drives water secretion (41). However, apical  $\text{K}^+$  currents of the intensity revealed in our experiments could contribute to the osmotic gradient by themselves by moving  $\text{K}^+$  out from the apical membrane, partially substituting for the paracellular movement of  $\text{Na}^+$ . Cook and Young (30) proposed that apical  $\text{K}^+$  secretion can favor  $\text{Cl}^-$  secretion, thereby bypassing some of the paracellular  $\text{Na}^+$  movement. We therefore tested a model (Fig. 5A) similar to that of Cook and Young they derived from measured values in dog trachea (30). The equivalent circuit of the model epithelium is shown in Fig. 5B. With this model, we show that apical  $\text{Cl}^-$  secretion increases by 50–300% when apical BK channels are activated (Fig. 5C). Even for very large apical  $\text{K}^+$  conductances, the luminal  $[\text{K}^+]$  does not increase to  $>20$ – $35$  mM (Fig. 5D), which is in the range found for secreting epithelia, especially airways (27).

**Effect of *KCNMA1* Knockdown on Apical, ATP-induced  $\text{K}^+$  Flux**—An shRNA approach was used to decrease *KCNMA1* (mRNA) expression. Fully differentiated cells infected with shRNA producing lentiviruses (as indicated in Table 2) revealed a significant reduction in relative *KCNMA1* (mRNA) expression in comparison with noninfected and cells infected with nontargeting virus (Fig. 6A). The same was true for BK  $\alpha$  subunit protein expression quantified on Western blots in the linear range (Fig. 6B). Furthermore, apical  $\text{K}^+$  currents in response to  $10 \mu\text{M}$  ATP in basolaterally permeabilized cells decreased significantly in cells with less *KCNMA1* (mRNA) expression (Fig. 6C). These data confirmed that functional expression of BK in the apical membrane contributed the majority of the measured,  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  current.

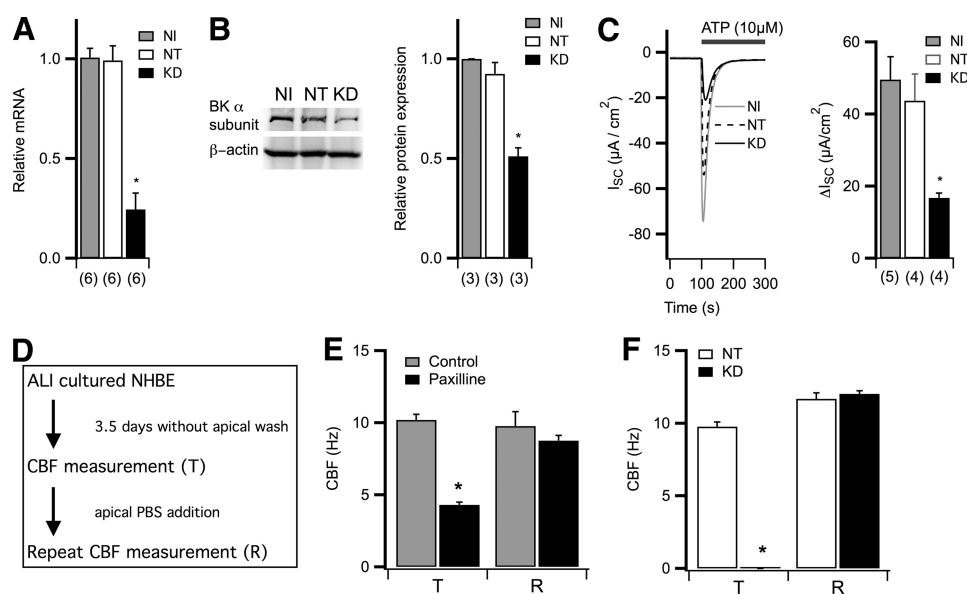
**ASL Volume Changes (as Assessed by Measuring CBF before and after ASL Volume Repletion) upon Chronic Inhibition of BK Activity**—Given the above data, the support of the computational model that apical  $\text{K}^+$  channels facilitate apical  $\text{Cl}^-$  secretion and our visual observation that during long term experiments with BK channel inhibitors and *KCNMA1* knockdown cells, the apical surface of the cells appeared dry and revealed no mucus transport, we wondered about the role of BK channels in ASL volume control. One important role of ion transport is to ensure adequate ASL volume necessary for mucociliary function. Decreased  $\text{Cl}^-$  secretion or increased  $\text{Na}^+$  absorption



**FIGURE 5. Activation of apical  $\text{K}^+$  channels increase  $\text{Cl}^-$  secretion in an epithelial cell simulation.** A, model from Cook and Young (30) for cation and  $\text{Cl}^-$  secretion, containing secondary active  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  transporters,  $\text{Na}^+/\text{K}^+$  ATPases, basolateral  $\text{K}^+$  channels, apical  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  channels, and a paracellular cation conductance, modified to also account for amiloride-sensitive epithelial sodium channel at the apical membrane. B, equivalent circuit for the model.  $E_i^K = RT/F \log([K]_i/[K]_o)$ ,  $E_i^{\text{Na}} = RT/F \log([Na]_i/[Na]_o)$ ,  $E_a^K = RT/F \log([K]_a/[K]_i)$ ,  $E_a^{\text{Na}} = RT/F \log([Na]_a/[Na]_i)$ ,  $E_c^{\text{Cl}} = -RT/F \log([Cl]_c/[Cl]_i)$ , and  $E_{ij}^K = RT/F \log([K]_i/[K]_j)$ . Current of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase is indicated by  $I_p$  in the drawing. C and D, simulations using the parameters in Table 3:  $\text{Cl}^-$  secretion (C) and luminal  $\text{K}^+$  concentrations (D) using the model in A with increasing apical  $\text{K}^+$  conductance. Dashed lines, no amiloride-sensitive epithelial sodium channel activity; solid lines, with amiloride-sensitive epithelial sodium channel activity at 35% of the  $\text{Cl}^-$  conductance. Two different paracellular cation resistances were used in the simulations,  $600 \Omega\text{cm}^2$  (black) and  $300 \Omega\text{cm}^2$  (red), to show the effect of the paracellular conductance on  $\text{Cl}^-$  secretion and luminal  $\text{K}^+$  concentration.

results in ASL volume depletion (classic example is cystic fibrosis). When ASL is depleted, the periciliary fluid height collapses (42). The periciliary fluid is required to be about as high as the length of cilia ( $7 \mu\text{m}$ ) to allow proper and effective ciliary beating and mucociliary transport (e.g. Ref. 43). Thus, low CBF that fully recovers after supplementing the apical compartment with fluid indicates ASL volume depletion (because direct effects on cilia would translate into abnormal CBF even after ASL volume repletion). Therefore, CBF was measured either in NHBE cells treated basolaterally with  $5 \mu\text{M}$  paxilline (or vehicle control) for 3.5 days or in *KCNMA1* knockdown cells (Fig. 6D). Paxilline was added basolaterally to avoid disturbing the apical compartment. As paxilline is cell membrane permeable, it will reach even the apical membrane in these long term experiments. During the 3.5 days of exposure, no apical washes were performed to avoid adding liquid to the apical surface. The same protocol was applied to nontargeting and *KCNMA1* knockdown cells.

In NHBE cells treated basolaterally for 3.5 days with paxilline, a significant decrease in CBF was observed compared with controls and mucus transport ceased. This decrease was immediately and fully reversible upon apical addition of Dulbecco's PBS (recovered cells; Fig. 6E); mucus transport was again apparent. After 3 days without any apical wash, NHBE cells in which *KCNMA1* was knocked down showed ciliostasis, making it



**FIGURE 6. *KCNMA1* knockdown decreases ATP-induced apical  $K^+$  movement and causes airway surface liquid volume loss.** *A–C*, shRNA knockdown of *KCNMA1* was accomplished by infection of nondifferentiated NHBE cells with lentiviruses collected after HEK293T transfection with pLKO.1 puromycin resistance encoding plasmids expressing a sequence targeting *KCNMA1* (mRNA) (NM\_001014797 and NM\_002247, TRC clone TRCN0000000212) (knockdown, *KD*) or a sequence that is nontargeting (*NT*; SHC002). Infected NHBE cells were selected with puromycin and grown until differentiation (~20 days). Noninfected (*NI*) cells were grown in parallel. *A*, expression of *KCNMA1* (mRNA) relative to GAPDH (mRNA) measured by qPCR, normalized to the average mRNA level in uninfected cells. The bars are reported as mean  $\pm$  S.E. of *n* experiments, indicated in parentheses. *B*, Western blot (left panel) and quantification (right panel) of BK  $\alpha$  (relative to  $\beta$ -actin) in fully differentiated NHBE cells (two lungs in triplicates). *C*, representative experiment (left panel) and quantification of the responses (right panel) of the effect of *KCNMA1* knockdown on apical  $K^+$  efflux using basolaterally permeabilized cells in Ussing chamber exposed to a basolateral to apical  $K^+$  gradient ( $I_{sc}$  measurements). *D–F*, sustained paxilline treatment and *KCNMA1* knockdown reduces ASL volume as assessed by CBF as a surrogate marker. Adequate ASL volume maintains an appropriate periciliary fluid height that allows normal ciliary activity. Thus, conditions that are associated with a CBF reduction that is fully rescued upon apical fluid addition indicate ASL volume depletion. *D*, summary of the protocol. Fully differentiated NHBE cells cultured at the ALI were apically not disturbed (no washes) for 3.5 days. Then, CBF was measured without disturbing the apical compartment (treated cells, *T*), upon which the cultures were apically supplemented with Dulbecco's PBS (recovered, *R*). *E*, effect of basolateral treatment of fully differentiated NHBE cells for 3.5 days with 5  $\mu M$  paxilline or equivalent volume of vehicle (control) on CBF (all  $n \geq 5$  cultures from three different lungs for each group). CBF decreased significantly in the paxilline-treated cells (*T*), but CBF fully recovered immediately after ASL volume repletion (*R*). *F*, effect of *KCNMA1* knockdown in comparison with nontargeting of fully differentiated NHBE cells on CBF after 3.5 days of leaving the apical compartment undisturbed. CBF in knockdown cells actually stopped (ciliostasis) but recovered fully immediately after ASL volume repletion (all  $n \geq 4$  for each group). An asterisk indicates significant difference by Tukey-Kramer comparison of all treatments after one-way analysis of variance ( $p < 0.001$ ).

impossible to measure CBF in these cultures (Fig. 6*F*). Mucus transport was also not present. However, beating was again completely restored (and mucus transport obvious) with apical Dulbecco's PBS addition (Fig. 6*F*). Recovery of ciliary beating to normal values by apical fluid addition in both experimental sets suggested that the CBF decrease was the result of ASL volume depletion, *i.e.* periciliary fluid collapse. Taken together, these results indicate that BK channels are involved in ASL volume control and thus mucociliary transport regulation.

## DISCUSSION

Our results strongly support the hypothesis that apical purinergic stimulation activates apical  $K^+$  secretion via BK channels and that blocking these BK channels causes ASL volume depletion. Apical nucleotide regulation of ASL volume is well recognized (19–21, 44–46); thus, the findings reported here are important for airway homeostasis and homeostasis of other epithelia that are regulated by purinergic actions.

Our data demonstrate that BK channels are functional at the apical membrane of primary NHBE cells, thereby playing a quantitatively important role in apical, ATP-stimulated  $K^+$  and anion secretion. The concept that apical  $K^+$  conductance can enhance anion secretion was first developed by Cook and Young (30) in 1989. In 1997, Clarke *et al.* (47) found an apical  $K^+$  current in human airway epithelia, suggesting that the

simultaneous secretion of both  $Cl^-$  and  $K^+$  will result in the transfer of ions and water directly to the airway lumen. Moser *et al.* (48) demonstrated apical localization and functional activity of several types of KCNQ channels in an airway epithelial cell line (Calu-3). More recently, Namkung *et al.* (27) measured apical  $K^+$  secretion in human airway bronchial epithelial cells and demonstrated the participation of apical KCNQ channels in  $K^+$  currents both at basal rates and during  $I_{sc}$  increases in response to 10  $\mu M$  forskolin; however, the magnitude of the current response to apical ATP was not examined, even though apical nucleotides are the physiological stimuli. More importantly, BK channel activity was not examined and thus not related to any of these reported apical currents. Finally, there are no reports that link defective apical  $K^+$  current to airway surface liquid depletion. Here, we show that BK channels are not only functional at the apical membrane but that their contribution to nucleotide-stimulated ion secretion and surface hydration is critical because inhibiting these channels disrupts the normal balance and causes ASL volume depletion and periciliary fluid height collapse.

Nucleotide-induced anion secretion in airway epithelia has been explored extensively (19–21, 44–46). Both ATP and UTP interact with the  $G_q$ -coupled  $P_2Y_2$  receptor and induce a fast mobilization of intracellular  $Ca^{2+}$  to the cytoplasm, which

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activates calcium-dependent channels, including BK and TMEM16. This ATP-stimulated pathway seems critical as seen in a recent paper reporting *TMEM16A* knock-out mice where ASL volume was inadequate (12), similar to our findings with knockdown of a channel upstream of TMEM16A (providing a  $\text{Cl}^-$  driving force). However, a recent paper challenged the notion that TMEM16A was the major calcium-activated chloride channel in human airway cells (49). Apical BK channels open in response to ATP or other stimuli. ATP is secreted by bronchial cells in response to several stimuli, even cell stretch experienced during quiet respiration (50, 51). Thus, ATP stimulation of airway epithelial cells occurs continuously and regulates ASL volume (19, 20, 46). At least part of the ATP exits the cell through apical pannexin 1 channels, which open in response to mechanical or hypo-osmolar stress (52) or in response to increased  $\text{Ca}^{2+}$  (52). ATP can also be released from mucin granules in goblet cells (53).

In addition to ATP-mediated activation, BK can be modulated by membrane stretch (54, 55),  $\text{H}_2\text{O}_2$  (56), arachidonic acid (57), phosphorylation (58), and other mechanisms (see Ref. 59 for review). This activation pattern may be important in the airway but remains unexplored here. In the human bronchial epithelial cell line 16HBE14, BK responds to hypotonic stress (60) and to high viscosity through TRPV4/BK interactions (61).

Our study shows that BK channels are functional at the apical membrane in fully differentiated human airway epithelia and that BK channels play a key role for the availability of adequate ASL volume and thereby for mucociliary function. According to our results and previous data, we propose a model where apical BK channels help drive the movement of water to the airway surface, responding to ATP signaling and mechanical stress. Failure of apical BK channel activity causes a loss of apical fluid availability and consequently mucociliary dysfunction. Thus, variations in BK channel expression or activity might be important in multiple airway diseases, including chronic bronchitis.

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### REFERENCES

- Orio, P., Rojas, P., Ferreira, G., and Latorre, R. (2002) *News Physiol. Sci.* **17**, 156–161
- Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschütz, R., and Pongs, O. (2000) *FEBS Lett.* **474**, 99–106
- Magleby, K. L. (2003) *J. Gen. Physiol.* **121**, 81–96
- Orio, P., and Latorre, R. (2005) *J. Gen. Physiol.* **125**, 395–411
- Lu, R., Alioua, A., Kumar, Y., Eghbali, M., Stefani, E., and Toro, L. (2006) *J. Physiol.* **570**, 65–72
- Sweet, T. B., and Cox, D. H. (2009) *J. Gen. Physiol.* **133**, 139–150
- Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., and Chou, J. L. (1989) *Science* **245**, 1066–1073
- Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, D. M., McCann, J. D., Klinger, K. W., Smith, A. E., and Welsh, M. J. (1990) *Nature* **347**, 358–363
- Caputo, A., Caci, E., Ferrera, L., Pedemonte, N., Barsanti, C., Sondo, E., Pfeffer, U., Ravazzolo, R., Zegarra-Moran, O., and Galiotta, L. J. (2008) *Science* **322**, 590–594
- Schroeder, B. C., Cheng, T., Jan, Y. N., and Jan, L. Y. (2008) *Cell* **134**,

- 1019–1029
- Yang, Y. D., Cho, H., Koo, J. Y., Tak, M. H., Cho, Y., Shim, W. S., Park, S. P., Lee, J., Lee, B., Kim, B. M., Raouf, R., Shin, Y. K., and Oh, U. (2008) *Nature* **455**, 1210–1215
- Rock, J. R., O'Neal, W. K., Gabriel, S. E., Randell, S. H., Harfe, B. D., Boucher, R. C., and Grubb, B. R. (2009) *J. Biol. Chem.* **284**, 14875–14880
- Galiotta, L. J., Zegarra-Moran, O., Mastrocola, T., Wöhrle, C., Rugolo, M., and Romeo, G. (1994) *Pflugers Arch.* **426**, 534–541
- Rugolo, M., Mastrocola, T., Wöhrle, C., Rasola, A., Gruenert, D. C., Romeo, G., and Galiotta, L. J. (1993) *J. Biol. Chem.* **268**, 24779–24784
- Mall, M., Wissner, A., Schreiber, R., Kuehr, J., Seydewitz, H. H., Brandis, M., Greger, R., and Kunzelmann, K. (2000) *Am. J. Respir. Cell Mol. Biol.* **23**, 283–289
- Cotton, C. U. (2000) *Am. J. Respir. Cell Mol. Biol.* **23**, 270–272
- Mall, M., Gonska, T., Thomas, J., Schreiber, R., Seydewitz, H. H., Kuehr, J., Brandis, M., and Kunzelmann, K. (2003) *Pediatr. Res.* **53**, 608–618
- Bernard, K., Bogliolo, S., Soriani, O., and Ehrenfeld, J. (2003) *J. Membr. Biol.* **196**, 15–31
- Lazarowski, E. R., Tarran, R., Grubb, B. R., van Heusden, C. A., Okada, S., and Boucher, R. C. (2004) *J. Biol. Chem.* **279**, 36855–36864
- Tarran, R., Trout, L., Donaldson, S. H., and Boucher, R. C. (2006) *J. Gen. Physiol.* **127**, 591–604
- Lazarowski, E. R., and Boucher, R. C. (2009) *Curr. Opin. Pharmacol.* **9**, 262–267
- Davis, C. W., and Lazarowski, E. (2008) *Respir. Physiol. Neurobiol.* **163**, 208–213
- Bernacki, S. H., Nelson, A. L., Abdullah, L., Sheehan, J. K., Harris, A., Davis, C. W., and Randell, S. H. (1999) *Am. J. Respir. Cell Mol. Biol.* **20**, 595–604
- Schmid, A., Bai, G., Schmid, N., Zaccolo, M., Ostrowski, L. E., Conner, G. E., Fregien, N., and Salathe, M. (2006) *J. Cell Sci.* **119**, 4176–4186
- Nlend, M. C., Bookman, R. J., Conner, G. E., and Salathe, M. (2002) *Am. J. Respir. Cell Mol. Biol.* **27**, 436–445
- Fragoso, M. A., Fernandez, V., Forteza, R., Randell, S. H., Salathe, M., and Conner, G. E. (2004) *J. Physiol.* **561**, 183–194
- Namkung, W., Song, Y., Mills, A. D., Padmawar, P., Finkbeiner, W. E., and Verkman, A. S. (2009) *J. Biol. Chem.* **284**, 15916–15926
- Willumsen, N. J., and Boucher, R. C. (1989) *Am. J. Physiol.* **256**, C1054–1063
- Gattas, M. V., Forteza, R., Fragoso, M. A., Fregien, N., Salas, P., Salathe, M., and Conner, G. E. (2009) *Free Radic. Biol. Med.* **47**, 1450–1458
- Cook, D. I., and Young, J. A. (1989) *J. Membr. Biol.* **110**, 139–146
- Lieb, T., Frei, C. W., Frohock, J. I., Bookman, R. J., and Salathe, M. (2002) *J. Physiol.* **538**, 633–646
- Salathe, M., and Bookman, R. J. (1999) *J. Physiol.* **520**, 851–865
- Wallner, M., Meera, P., and Toro, L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 4137–4142
- Xia, X. M., Ding, J. P., and Lingle, C. J. (2003) *J. Gen. Physiol.* **121**, 125–148
- Sun, X., Zhou, D., Zhang, P., Moczydlowski, E. G., and Haddad, G. G. (2007) *J. Neurophysiol.* **97**, 62–69
- Xia, X. M., Ding, J. P., Zeng, X. H., Duan, K. L., and Lingle, C. J. (2000) *J. Neurosci.* **20**, 4890–4903
- Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000) *J. Biol. Chem.* **275**, 6453–6461
- Sun, X. P., Yazejian, B., and Grinnell, A. D. (2004) *J. Physiol.* **557**, 207–228
- Morimoto, T., Sakamoto, K., Sade, H., Ohya, S., Muraki, K., and Imaizumi, Y. (2007) *Mol. Pharmacol.* **71**, 1075–1088
- Sanchez, M., and McManus, O. B. (1996) *Neuropharmacology* **35**, 963–968
- Begenisich, T., and Melvin, J. E. (1998) *J. Membr. Biol.* **163**, 77–85
- Tarran, R., Grubb, B. R., Gatzky, J. T., Davis, C. W., and Boucher, R. C. (2001) *J. Gen. Physiol.* **118**, 223–236
- Tarran, R., Button, B., Picher, M., Paradiso, A. M., Ribeiro, C. M., Lazarowski, E. R., Zhang, L., Collins, P. L., Pickles, R. J., Fredberg, J. J., and Boucher, R. C. (2005) *J. Biol. Chem.* **280**, 35751–35759
- Paradiso, A. M., Ribeiro, C. M., and Boucher, R. C. (2001) *J. Gen. Physiol.* **117**, 53–67
- Faria, D., Schreiber, R., and Kunzelmann, K. (2009) *Pflugers Arch.* **457**, 1373–1380

46. Tarran, R., Button, B., and Boucher, R. C. (2006) *Annu. Rev. Physiol.* **68**, 543–561
47. Clarke, L. L., Chinet, T., and Boucher, R. C. (1997) *Am. J. Physiol.* **272**, L1084–1091
48. Moser, S. L., Harron, S. A., Crack, J., Fawcett, J. P., and Cowley, E. A. (2008) *J. Membr. Biol.* **221**, 153–163
49. Namkung, W., Phuan, P. W., and Verkman, A. S. (2011) *J. Biol. Chem.* **286**, 2365–2374
50. Button, B., and Boucher, R. C. (2008) *Respir. Physiol. Neurobiol.* **163**, 189–201
51. Lazarowski, E. R., Boucher, R. C., and Harden, T. K. (2003) *Mol. Pharmacol.* **64**, 785–795
52. Ransford, G. A., Fregien, N., Qiu, F., Dahl, G., Conner, G. E., and Salathe, M. (2009) *Am. J. Respir. Cell Mol. Biol.* **41**, 525–534
53. Kreda, S. M., Okada, S. F., van Heusden, C. A., O'Neal, W., Gabriel, S., Abdullah, L., Davis, C. W., Boucher, R. C., and Lazarowski, E. R. (2007) *J. Physiol.* **584**, 245–259
54. Kirber, M. T., Ordway, R. W., Clapp, L. H., Walsh, J. V., Jr., and Singer, J. J. (1992) *FEBS Lett.* **297**, 24–28
55. Hammami, S., Willumsen, N. J., Olsen, H. L., Morera, F. J., Latorre, R., and Klaerke, D. A. (2009) *J. Physiol.* **587**, 2225–2231
56. Liu, B., Gan, L., Sun, X., Zhu, Y., Tong, Z., Xu, H., and Yang, X. (2009) *Biochim. Biophys. Acta* **1788**, 2174–2182
57. Lu, T., Wang, X. L., He, T., Zhou, W., Kaduce, T. L., Katusic, Z. S., Spector, A. A., and Lee, H. C. (2005) *Diabetes* **54**, 2155–2163
58. Zhou, X. B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., Ruth, P., and Korth, M. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 8005–8010
59. Hou, S., Heinemann, S. H., and Hoshi, T. (2009) *Physiology* **24**, 26–35
60. Fernández-Fernández, J. M., Nobles, M., Currid, A., Vázquez, E., and Valverde, M. A. (2002) *Am. J. Physiol. Cell Physiol.* **283**, C1705–1714
61. Fernández-Fernández, J. M., Andrade, Y. N., Arniges, M., Fernandes, J., Plata, C., Rubio-Moscardo, F., Vázquez, E., and Valverde, M. A. (2008) *Pflugers Arch.* **457**, 149–159
62. Welsh, M. J. (1983) *J. Clin. Invest.* **71**, 1392–1401
63. Welsh, M. J., Smith, P. L., and Frizzell, R. A. (1983) *J. Membr. Biol.* **71**, 209–218