Regulation of ENaC and CFTR expression with K⁺ channel modulators and effect on fluid absorption across alveolar epithelial cells

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In a recent study (Leroy C, Dagenais A, Berthiaume Y, and Brochiero E. Am J Physiol Lung Cell Mol Physiol 286: L1027–L1037, 2004), we identified an ATP-sensitive K⁺ (KATP) channel in alveolar epithelial cells, formed by inwardly rectifying K⁺ channel Kir6.1/sulfonurea receptor (SUR)2B subunits. We found that short applications of KATP, voltage-dependent K⁺ channel KvLQT1, and calcium-activated K⁺ (KCa) channel modulators modified Na⁺ and Cl⁻ currents in alveolar monolayers. In addition, it was shown previously that a KATP opener increased alveolar liquid clearance in human lungs by a mechanism possibly related to epithelial sodium channels (ENaC). We therefore hypothesized that prolonged treatment with KATP channel modulators could induce a sustained regulation of ENaC activity and/or expression. Alveolar monolayers were treated for 24 h with inhibitors of KATP, KvLQT1, and KCa channels identified by PCR. Glibenclamide and clofilium (KvLQT1 and Kir6.1/SUR2B subunits) strongly reduced basal transepithelial current, whereas pinacidil, a KATP activator, increased them. Interestingly, K⁺ inhibitors or membrane depolarization (induced by valinomycin in high-K⁺ medium) decreased α-, β-, and γ-ENaC and CFTR mRNA α-ENaC and CFTR proteins also declined after glibenclamide or clofilium treatment. Conversely, pinacidil augmented ENaC and CFTR mRNAs and proteins. Since alveolar fluid transport was found to be driven, at least in part, by Na⁺ transport through ENaC, we tested the impact of K⁺ channel modulators on fluid absorption across alveolar monolayers. We found that glibenclamide and clofilium reduced fluid absorption to a level similar to that seen in the presence of amiloride, whereas pinacidil slightly enhanced it. Long-term regulation of ENaC and CFTR expression by K⁺ channel activity could benefit patients with pulmonary diseases affecting ion transport and fluid clearance.

It has been well established that fluid absorption and secretion across alveolar or tracheobronchial epithelia (2, 7, 8, 38) are mainly driven by transepithelial transport of Na⁺ and Cl⁻. Effective control of Na⁺ and Cl⁻ transepithelial transport is then essential for periciliary volume and mucociliary clearance in airways, processes that are dysfunctional in cystic fibrosis (7, 8). In alveoli, Na⁺ and Cl⁻ transport in alveolar epithelial type II (5) and type I (26) cells is also essential for fluid absorption at birth as well as in adult lungs for the resolution of pathological conditions such as pulmonary edema. Cl⁻ secretion via the CFTR (20), Na⁺ transport through epithelial sodium channels (ENaC) (37, 42), and liquid clearance (3, 6, 25) can be stimulated by pharmacological agents such as β-adrenergic agonists and cAMP analogs. However, it was recently shown that Na⁺ and Cl⁻ transport can also be controlled by K⁺ channel activity. Indeed, we recently demonstrated (31) the presence of an ATP-sensitive K⁺ (KATP) channel, formed by inwardly rectifying K⁺ channel Kir6.1 and sulfonurea receptor SUR2B subunits, in alveolar epithelial cells. Acute application of KATP modulators can control Na⁺ and Cl⁻ transport. In addition, a previous study (53) revealed that treatment with YM-934, a KATP channel opener, increased alveolar liquid clearance in human lungs (53). It was also observed that glibenclamide and amiloride inhibited this response. These results suggested that KATP activation might stimulate Na⁺ transport in the lungs, which has been identified as the main mechanism involved in alveolar liquid clearance (4). However, sustained increased Na⁺ transport in the alveolar epithelium by long-term treatment with KATP activators has never been directly demonstrated, and the mechanisms of activation have not been clearly defined.

Other classes of K⁺ channels could be important for fluid secretion and absorption in alveolar epithelial cells, since they have been found to modulate ion transport in airway epithelia. Indeed, voltage-dependent K⁺ KvLQT1 channels and calcium-activated K⁺ (IKCa) channels have been identified as key elements of ion transport. A severe reduction of Cl⁻ secretion has been reported after inhibition of KvLQT1 and intermediate-conductance, calcium-activated K⁺ (IKCa) channels in nasal and bronchial cells (12, 34, 35). More interestingly, it has been observed that IKCa channel openers stimulate Cl⁻ secretion in Calu-3 cells (16, 56), in primary cultures of human bronchial epithelium (56), and in nasal cells (35). It has, therefore, been proposed that these agents could constitute a promising strategy to stimulate Cl⁻ secretion, via residual CFTR channels or alternative calcium-activated Cl⁻ channels (35), in cystic fibrosis. These KvLQT1 (14, 23, 36) and calcium-activated K⁺ channels [high-conductance KCa (maxi-KCa, Slo1; Refs. 28, 29) and IKCa (Refs. 12, 39)] are well distributed in the upper airways. However, their presence was not clearly demonstrated in alveolar epithelia, even if our recent results (31) indicated their activity in alveolar epithelial cells in primary culture.
The aim of our study was first to verify the presence of KvLQT1 and KCa channels and to determine their molecular identity in alveolar epithelial cells. Their expression in freshly isolated and cultured alveolar cells was also compared. Second, we tried to establish whether long-term treatment with modulators of KvLQT1, KCa, and KATP channels could induce sustained regulation of Na⁺ and Cl⁻ currents as well as ENaC and CFTR expression in cultured alveolar cells. Finally, we studied the impact of Na⁺ and Cl⁻ transport regulation by K⁺ channel modulators on fluid transport across alveolar epithelial cell monolayers.

MATERIALS AND METHODS

Alveolar Epithelial Type II Cell Isolation and Primary Culture

Alveolar epithelial type II cells were isolated from male Sprague-Dawley rats according to a well-established protocol (22, 31). In brief, the lungs were washed to remove blood cells and alveolar macrophages before treatment with elastase. They were then minced, and the resulting suspensions were filtered. Alveolar cells were collected and purified by a differential adherence technique (17), which enhances the purity of the alveolar type II cell pool to 86% (10, 31). This freshly isolated cell suspension was used directly or cultured in Eagle’s minimum essential medium (MEM)-FBS medium on Costar Transwell permeant filters (4 cm², Costar Transwell, Toronto, ON, Canada; 1 × 10⁶ cells/cm²), as described previously (31).

Molecular Biology

RNA purification. Total RNA from alveolar epithelial cells (freshly isolated or cultured on filters for 1–4 days) was purified with TRIzol reagent according to the manufacturer’s instructions (Invitrogen, Burlington, ON, Canada).

Polymerase chain reaction amplification of ionic channels. Five micrograms of total RNA, purified from freshly isolated or cultured alveolar epithelial cells, was reverse-transcribed to cDNA with MMLV reverse transcriptase. Five micrograms of total RNA, purified from freshly isolated or cultured alveolar cells, was reverse-transcribed to cDNA with MMLV reverse transcriptase. Five micrograms of total RNA, purified from freshly isolated or cultured alveolar cells, was reverse-transcribed to cDNA with MMLV reverse transcriptase. Five micrograms of total RNA, purified from freshly isolated or cultured alveolar cells, was reverse-transcribed to cDNA with MMLV reverse transcriptase.

Sequencing. The 462-bp PCR product (reverse primer pair [sense 5'-ENaC (sense 5'-CA-AGGCTTGCCGTTGAG-3'), antisense 5'-GCGTATGCAAGGAAAC-3'], 1 μM final concentration of each, for Kir6.1 and maxi-KCa) and once in TBS for 15 min and then incubated with the secondary antibody anti-rabbit IgG (1:2,000; Bio-Rad, Mississauga, ON, Canada) and rinsed with TBS-Tween 200. The membranes were dried with a stream of air, exposed to Hyperfilm ECL (Amersham), and the bands were visualized by autoradiography.

Immunoblotting

Total proteins were extracted from alveolar cells (obtained from at least 5 different rats), cultured on permeant filters for 3 days, and treated or not with K⁺ channel modulators for an additional 24 h before analysis. After the monolayers were washed twice with PBS, the cells were detached by gentle scraping, and the suspension was centrifuged at 2,800 g at 4°C. For CFTR detection, proteins were extracted in RIPA buffer, denatured on ice in sample buffer A (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 125 mM DTT), and then separated by polyacrylamide gel electrophoresis (6.5%) (10). CFTR protein of alveolar extract (120 μg) was detected with an anti-CFTR primary antibody (1:500, H-182; Santa Cruz Biotechnology). Specificity of the antibody was confirmed (10) in BHK cells transfected with CFTR (a gift from Dr. J. Hanrahan, McGill University, Montreal, QC, Canada) and NDu1 (kindly provided by Dr. J. Zabner, University of Iowa, Iowa City, IA) as positive controls (175-kDa band). No band could be detected with BHK cells stably transfected with empty pNUT vector (negative control; a gift from Dr. J. Hanrahan). In alveolar cells, the observed 164-kDa band was consistent with mature rat CFTR (150–165 kDa) (44).

An adapted immunoblotting protocol was adopted for α-ENaC, Kir6.1, KvLQT1, IKCa, and maxi-KCa detection. The cell pellet was solubilized in lysate solution [150 mM NaCl, 50 mM Tris·HCl, pH 7.6, 1% Triton X-100, 0.1% SDS, protease cocktail (Complete Mini EDTA-free protease inhibitor cocktail, Roche, Mannheim, Germany)] for 1 h on ice and centrifuged at 12,000 g for 5 min. The supernatants were collected, and protein contents were estimated by Coomassie blue assay (Bradford, Pierce, Rockford, IL) with BSA as standard. Proteins were then solubilized at 95°C for 5 min in sample buffer B (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, 4% β-mercaptoethanol). Proteins (50 μg for α-ENaC, 40 μg for Kir6.1 and KvLQT1, 120–135 μg for IKCa, and maxi-KCa) were separated by polyacrylamide gel electrophoresis (7.5%) and transferred onto polyvinylidene difluoride membranes. The blots were first blocked with 5% milk in Tris-buffered saline (TBS)-TWEEN (TBST; 500 mM NaCl, 20 mM Tris·HCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature or overnight at 4°C and then stained with commercial anti-α-ENaC antibody (dilution 1:1,000, PA1-920; Affinity BioReagents, Golden, CO), anti-Kir6.1 (dilution 1:200, sc-11224, Santa Cruz Biotechnology), anti-KvLQT1 (dilution 1:1,000, sc-10645, Santa Cruz Biotechnology), anti-IKCa (dilution 1:300, P4997, Sigma-Aldrich), or anti-maxi-KCa (dilution 1:200, APC-021, Alomone Labs, Jerusalem, Israel) in TBST plus 10% milk for 16 h at 4°C. The membranes were washed three times in TBST for 15 min and once in TBS for 15 min and then incubated with the secondary antibody anti-rabbit IgG (1:2,000; Bio-Rad, Mississauga, ON, Canada).

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 ada; for ENaC, IKCa, and maxi-IKCa) or with anti-goat (1:4,000; Santa Cruz Biotechnology; for Kir6.1 and KvLQT1) linked to horseradish peroxidase in TBST plus 10% milk for 1 h at room temperature. They were washed three times in TBST for 15 min and once in TBS for 15 min before development (for 5 min) with chemiluminescence reagent (ECL Plus; Amersham, Little Chalfont, UK). Finally, a diagnostic film (Kodak, Rochester, NY) was exposed with the membranes for 10 s up to 3 min. The anti-α ENaC antibody (PA1-920) recognized a 83- to 90-kDa band in alveolar epithelial cell extracts (13, 61). The specificity of this antibody was verified with its blocking peptide (PA1-920-neutralizing peptide PEP-088, Affinity BioReagents). In addition, protein extracts (a gift from Dr. D. Rotin, Sick Children’s Hospital, Toronto, ON, Canada) from untransfected Madin-Darby canine kidney (MDCK) cells (1 µg of protein) and transfected MDCK cells with rat α ENaC tagged with the influenza hemagglutinin epitope (1 µg of protein) were negative and positive controls, respectively (~97 KDa). The anti-Kir6.1, anti-KvLQT1, and anti-IKCa antibodies recognized ~50- (54), 75- (32), and ~55-kDa bands, respectively. The specificity of anti-Kir6.1 and anti-KvLQT1 antibodies was verified with their respective blocking peptides (sc-11224P and sc10645P, Santa Cruz Biotechnology). Unfortunately, the specificity of the 55-kDa band detected with anti-IKCa antibody was not verified because the blocking peptide (Sigma-Aldrich) was unavailable.

Electrophysiology

Alveolar epithelial cells were cultured on filters (Costar Transwell, 4 cm²) for 3 days until they formed a tight epithelium (≥1,200 Ω-cm²). The monolayers were then treated for 24 h with K⁺ channel modulators. The electrophysiological characteristics of alveolar monolayers were studied with an epithelial voltohmmeter (EVOM; World Precision Instruments, Sarasota, FL; Ref. 13) or by short-circuit current (Isc) measurements in a Ussing chamber (31). The EVOM successively recorded potential differences (PD, mV) generated by the cell monolayers and transepithelial resistance (Rt, Ω-cm²) across the cell monolayers. Transepithelial current (Isc) across the monolayers was then calculated according to the following formula:

\[ \text{Isc} = \frac{\text{PD}}{\text{Rt}} \]  

(13). To quantify the amount of amiloride-sensitive current generated by intact alveolar monolayers treated for 24 h with or without K⁺ channel inhibitors, Isc was measured successively before and 5 min after incubation with 1 µM amiloride (apical side). The monolayers were then treated with 10 µM forskolin (basolateral side) for a 55-min period in the presence of amiloride to quantify the effect of increasing cAMP levels on Cl⁻ currents.

For Isc measurements, alveolar monolayers were mounted in a heated (37°C) Ussing chamber and perfused on the apical and basolateral sides with warm normal physiological solution (containing in mM: 141 NaCl, 5.4 KCl, 0.78 NaH2PO4, 0.8 MgCl2, 1.8 CaCl2, 5 glucose and 15 HEPES, pH 7.4). Transepithelial PD was clamped to zero by an external voltage-clamp amplifier (VCCM2, Physiological Instruments) with KCl agar-calomel half-cells and Ag-AgCl electrodes, and the resulting Isc was recorded continuously on a computer with a PowerLab system (ADInstruments, Toronto, ON, Canada; Ref. 31). To quantify the amount of active ENaC and CFTR channels at the apical membrane after treatment with K⁺ channel modulators, Isc was measured after establishment of a Na⁺ or Cl⁻ gradient and permeabilization of the basolateral membrane with amphotericin B (7.5 µM). The apical-to-basolateral Na⁺ gradient was created by bathing the apical side with normal physiological solution and replacing 116 mM NaCl by an equivalent amount of NMDG-Cl⁻ for the basolateral side. A basolateral-to-apical Cl⁻ gradient was established with a normal physiological solution at the basolateral side and a low-Cl⁻ solution at the apical side (116 mM NaCl replaced by 116 mM Na gluconate).

Fluid Transport Across Alveolar Epithelial Cell Monolayers

Fluid transport across alveolar monolayers was measured with 125I-labeled albumin as a volume marker, following a method adapted from Fang et al. (21). Twenty-four and forty-eight hours after seeding, the culture medium at the apical side of the alveolar epithelial monolayers was removed to create an air-liquid interface (ALI). At day 4, 1.5 ml of culture medium (MEM-10% FBS) containing 0.5 Ci/ml 125I-albumin was added to the apical side of the monolayers. At the same time, the monolayers were treated with ion channel modulators. Amiloride (10 µM) was added at the apical side in 125I-albumin-containing MEM. Clofilium (5 µM), glibenclamide (100 µM), or pinacidil (100 µM) was applied at the basolateral side of the monolayers. The cells were then incubated at 37°C with 5% CO₂ in an humidified incubator. After 5 min (t5), 250 µl of the apical medium was collected (corresponding to the initial sample). Monolayers were then laid in the incubator for a 24-h period, when a second 250-µl aliquot was collected (t24). The t5 and t24 samples were weighed and counted in a gamma counter (Cobra Auto-gamma, Packard) for 5 min. Fluid transport was calculated according to the following formula: fluid absorption (in %) = [1 – (cpm of t5 sample/weight of t5 sample)/(cpm of t24 sample/weight of t24 sample)] × 100, where cpm is counts/minute. The absorbed volume was then calculated in microliters per square centimeter per hour, and the results are presented as percentage of fluid absorption across control untreated monolayers. Permeability to protein (Pp) was also estimated in each monolayer. The remaining apical and basolateral media were collected; the monolayers were washed with H₂O₂, and the washing medium was collected and counted. Pp of each monolayer was calculated according to the following formula: Pp = (cpm of basolateral compartment)/(cpm of apical compartment), where cpm of the apical compartment was cpm of the t5 sample + cpm of the t24 sample + cpm of the remaining apical volume + cpm of the apical washing solution. Mean Pp was 0.57 ± 0.05%; monolayers with Pp >3% were discarded. Free 125I measured in the presence of trichlo-roacetic acid (20%) was <2.5%. Evaporative loss of fluid measured in an empty, Parafilm-sealed Transwell membrane was null.

Statistics

Average values are given as means ± SE; n represents the number of experiments that were performed on at least four different animals. Comparisons between groups were made by one-group or paired t-test with Statview software (SAS Institute, Cary, NC). A probability of P < 0.05 was considered to be significant.

RESULTS

Molecular Identity and Expression of K⁺ Channels in Alveolar Epithelial Cells

IKCa and Slo1 primer pairs, designed from intermediate- and high-conductance calcium-activated K⁺ channels, respectively, amplified 500- and 461-bp products from the cDNA of freshly isolated alveolar cells, as expected (Fig. 1A). In addition, a 462-bp product could be ampliﬁed with the KvLQT1 primer pair. These 500-, 461-, and 462-bp bands were excised and cloned in pBluescript SKII (Stratagene, La Jolla, CA). The results demonstrated the presence of KvLQT1 mRNA as well as IKCa and Slo1 mRNAs coding for intermediate- and large-conductance KCa channels, respectively, in alveolar epithelial cells.
Fig. 1. Molecular identity and expression of K⁺ channels in freshly isolated and cultured alveolar epithelial cells. A: agarose gels showing RT-PCR products amplified from freshly isolated rat alveolar epithelial cell cDNA with PCR primer pairs designed from cloned rat intermediate-conductance calcium-activated K⁺ (IKCa) channel, rat maxi-conductance KCa (Slo1), and voltage-dependent K⁺ channel KvLQT1. Primers are described in MATERIALS AND METHODS. M, marker. B: densitometric semiquantification of KvLQT1, Slo, and IKCa cDNA expression from freshly isolated alveolar epithelial cells (time 0) and alveolar monolayers in primary culture for 1, 2, 3, and 4 days, normalized with β-actin expression and shown as % of the signal observed on day 0 (n = 5). C: detection of KvLQT1 (KvLQT1-Ab) and inwardly rectifying K⁺ channel Kir6.1 (Kir6.1-Ab) proteins by Western blot analysis of a representative (from at least 5 different animals) epithelial alveolar cell lysate (cultured for 4 days on permeant filters, 40 μg protein). Specificity of the antibodies was confirmed with their neutralizing peptide (KvLQT1-Ab + peptide, Kir6.1-Ab + peptide).

Since we recently found (31) that expression of the Kir6.1 and SUR2B subunits (forming the alveolar KATP channel) changed as a function of time of culture (initial decrease between days 0 and 2, followed by an increase on days 3 and 4), we decided to track and compare the expression of IKCa, Slo1, and KvLQT1 mRNA in culture. As observed in Fig. 1B, KvLQT1 expression decreased by 29 ± 7% (P = 0.015) between freshly isolated (day 0) alveolar cells and alveolar cell monolayers cultured on filters until day 4 (when the electrophysiological experiments were performed). Conversely, there was a nonsignificant increase of IKCa and Slo1 expression between days 0 and 4.

Expression of KvLQT1, Kir6.1, IKCa, and Slo1 at the protein level was then explored. Goat polyclonal antibodies raised against peptides mapping near the COOH terminus of KvLQT1 or Kir6.1 proteins detected 75- and ~50-kDa bands, the expected molecular mass of KvLQT1 and Kir6.1, respectively (Fig. 1C). The specificities of these antibodies were verified with their respective neutralizing peptides (Fig. 1C). The rabbit anti-IKCa antibody detected several bands, one of them at ~55 kDa, the expected mass of IKCa (data not shown). However, the specificity of this band could not be verified because the blocking peptide was unavailable. Finally, an anti-rabbit maxi-KCa (Slo1) antibody did not detect any band in alveolar cell extracts (50- to 120-μg proteins).

Effect of Long-Term Treatment with K⁺ Channel Modulators on Transepithelial Currents in Alveolar Epithelial Cells

Effect of long-term treatment with KATP, KvLQT1, and KCa inhibitors in intact alveolar monolayers. We have already shown (31) that short-term treatment with KATP, KvLQT1, and KCa channel inhibitors reduced Na⁺ and Cl⁻ currents in alveolar epithelial cell monolayers. We now tried to determine whether prolonged application of K⁺ channel inhibitors (24-h treatment) could induce sustained modulation of Na⁺ and Cl⁻ currents.

In nontreated monolayers, mean basal Iₑ was 5.0 ± 0.1 μA/cm² (n = 97). It was significantly reduced by 60% and 68% (P < 0.0001) by 24-h treatment with glibenclamide (100 μM, basolateral side; an inhibitor of KATP; Fig. 2A; n = 14) and clofilium (5 μM, basolateral side; an inhibitor of KvLQT1; Fig. 2D; n = 14), respectively. Clotrimazole (20 μM), an inhibitor of IKCa channels, also significantly decreased basal Iₑ (25% inhibition, P < 0.0001; n = 18). Other inhibitors of KCa channels, charybdotoxin (200 nM; an inhibitor of maxi-KCa and IKCa channels), Tram34 (5 μM; an inhibitor of IKCa channels), and iberiotoxin (100 nM; an inhibitor of maxi-KCa channels), elicited nonsignificant declines of basal Iₑ.

Treatment with K⁺ channel inhibitors also influenced amiloride-sensitive Na⁺ currents. In control monolayers, 65 ± 1% of basal Iₑ was sensitive to amiloride (Iₑ-amil of 3.2 ± 0.1
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µA/cm²; n = 97). I_{te-amil} was decreased by 74% (P = 0.0003, n = 14) and 75% (P < 0.0001, n = 14) with glibenclamide (Fig. 2B) and clofilium (Fig. 2F), respectively. Like basal I_{te}, I_{te-amil} was poorly reduced by K_{Ca} inhibitors; only clotrimazole significantly diminished I_{te-amil} by 29% (P < 0.0001; n = 18).

Finally, the impact of K⁺ channel inhibitors was tested on cAMP-stimulated Cl⁻ currents. After ENaC inhibition by amiloride, elevation of cAMP levels with forskolin (10 µM, basolateral side) elicited a progressive increase in I_{sc} from 1.7 ± 0.1 to 4.1 ± 0.1 µA/cm² after 1 h (i.e., 143.8 ± 3.8% rise; n = 97). This forskolin-induced Cl⁻ current (I_{te-Fk}; 2.4 ± 0.1 µA/cm²) was totally suppressed by 5-nitro-2-(3-phenylpropy1amino)benzoic acid (NPPB), an inhibitor of anion conductance. I_{te-Fk} was also reduced by 41% (P = 0.0012, n = 14; Fig. 2C) and 46% (P = 0.0001, n = 14; Fig. 2F) by basolateral glibenclamide and clofilium, respectively. The effect of glibenclamide was specifically due to the inhibition of basolateral K_{ATP} channels, since apical treatment with glibenclamide (100 µM, 24 h) had no significant effect on basal I_{te}, I_{te-amil}, and I_{te-Fk} (data not shown). Some K_{Ca} channel inhibitors, more precisely charybotoxin, iberitoxin, clotrimazole, and Tram34, inhibited I_{te-Fk} by 13% (P = 0.028, n = 20), 11% [not significant (NS), n = 14], 18% (P = 0.0005, n = 18), and 22% (P = 0.04, n = 10), respectively.

Effect of long-term K⁺ channel inhibition on amiloride-sensitive currents in basolaterally permeabilized alveolar monolayers. The impact of long-term inhibition of K⁺ channel on the presence of active ENaC channels at the apical membrane was then evaluated in an Ussing chamber after permeabilization of the basolateral membrane of alveolar monolayers (Fig. 3). In these experiments, an apical-to-basolateral Na⁺ gradient was created, and the basolateral membrane was permeabilized by amphotericin B (7.5 µM) (Fig. 3A). Na⁺ currents, I_{amil}, sensitive to 1 µM amiloride [ENaC highly selective channel (HSC) currents] and to subsequent addition of 10 µM amiloride [nonselective channel (NSC) currents] were then compared in nontreated and clofilium-treated monolayers. The 1-µM amiloride-sensitive ENaC current was reduced by 57% after clofilium treatment (P = 0.028, n = 5; Fig. 3B), whereas current sensitive to the subsequent addition of 10 µM amiloride was not modified, indicating that clofilium application mainly affected the number of active ENaC channels.

Effect of long-term treatment with pinacidil, an activator of K_{ATP} channels, on alveolar transepithelial currents. Since it has been shown that prolonged activation of K_{ATP} channels increased liquid clearance in human lungs by a mechanism sensitive to amiloride (53), we measured the effect of pinacidil on Na⁺ transport across alveolar monolayers in an Ussing chamber (Fig. 4A). Basal I_{sc} was significantly augmented by pinacidil treatment (6.6 ± 0.6 and 7.9 ± 0.5 µA/cm² in the absence and presence of pinacidil, respectively, i.e., a mean difference of 1.3 µA/cm²; P < 0.005). In addition, I_{amil} increased from 4.3 ± 0.5 µA/cm² (control condition) to 5.3 ± 0.4 µA/cm² in 24-h pinacidil-treated monolayers, i.e., a 23% increment (P < 0.02).

Conversely, forskolin-stimulated and NPPB-sensitive Cl⁻ currents were not modified by pinacidil treatment in intact alveolar monolayers (Fig. 4A). However, it must be noted that the kinetics of Cl⁻ current stimulation were slow, and the amplitude of the current was low in intact monolayers (Fig. 4A). We then hypothesized that Cl⁻ transepithelial current could be rate limited by the basolateral membrane (due to low activity of Na⁺-K⁺-2Cl⁻ cotransport, for example). To test this hypothesis, the basolateral side of alveolar monolayers was permeabilized and a basolateral-to-apical Cl⁻ gradient was created. The addition of forskolin (10 µM; apical side) then induced a faster and higher increase of I_{sc} (an increment of 10.3 ± 1.4 µA/cm² after 10 min; n = 17; Fig. 4B) in permeabilized monolayers compared with intact monolayers...
(2.4 μA/cm² after 50 min; Fig. 4A). This cAMP-stimulated and NPPB-sensitive Cl⁻ current was significantly enhanced by pinacidil treatment (mean difference of 2.6 μA/cm² in the absence and presence of pinacidil; n = 17, P < 0.04) (Fig. 4C).

Regulation of ENaC and CFTR mRNA and Protein Expression by K⁺ Channel Modulators

Since long-term treatment with K⁺ channel modulators regulated Na⁺ and Cl⁻ currents, we tested the hypothesis that they also modify ENaC and CFTR expression.

Effect of K⁺ channel modulators on ENaC expression. Twenty-four-hour treatment with glibenclamide (100 μM, basolateral side) reduced α-ENaC expression by 29 ± 7% (P = 0.005, n = 8; Fig. 5A) as measured by semiquantitative RT-PCR. This effect seemed specific to basolateral K_ATP channels since apical treatment with glibenclamide failed to decrease α-ENaC expression (increase of 1.5 ± 10.9%; NS,

Fig. 3. Amiloride-sensitive short-circuit current (Isc) after amphotericin B permeabilization of the basolateral membrane. Alveolar epithelial cells were cultured for 3 days on permeant filters and treated or not for an additional 24 h with clofilium (5 μM, basolateral side; n = 5). The filters were then washed and mounted in an Ussing chamber and bathed with an asymmetric physiological solution (normal physiological solution at the apical side and low-Na⁺ physiological solution at the basolateral side, where 82% of Na⁺ was replaced by NMDG-Cl⁻). On stabilization of the current (10 min), the basolateral membrane was permeabilized by perfusion of the lower chamber with the same low-Na⁺ solution containing 7.5 μM amphotericin B. When the current reached a new plateau, 1 μM amiloride and then 10 μM amiloride were added at the apical side. A: mean I沈 currents measured on nonpretreated (n = 5) alveolar monolayers. B: amiloride-sensitive I沈 (Iamil 1 μM: ISc sensitive to 1 μM; Iamil 1–10 μM: additional decrease in ISc following additional 10 μM amiloride) were then compared in nontreated (n = 5) and clofilium-treated (5 μM, basolateral side for 24 h; n = 5) monolayers. ENaC activity at the apical membrane, estimated by Iamil 1 μM, was significantly reduced by clofilium treatment.

Fig. 4. Effect of pinacidil, an activator of K_ATP channels, on transepithelial transport. A: mean ISc values measured in an Ussing chamber on nontreated (control; n = 11) and pinacidil-treated (100 μM, basolateral side, for 24 h; n = 11) intact alveolar monolayers. After stabilization of basal ISc, amiloride (1 μM, apical side) was applied. Forskolin (10 μM, basolateral side) was subsequently added to stimulate cAMP-stimulated Cl⁻ currents, inhibited by 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; 200 μM, apical side) at the end of the experiment. B: filters were bathed with an asymmetric physiological solution (normal physiological solution at the basolateral side and low-Cl⁻ physiological solution at the apical side, where 77% of Cl⁻ was replaced by gluconate). On stabilization of the current (10 min), the basolateral membrane was permeabilized by perfusion of the lower chamber with normal physiological solution containing 7.5 μM amphotericin B. Amiloride (1 μM, apical side), forskolin (10 μM, apical side), and then NPPB (200 μM, apical side) were applied successively. C: cAMP-stimulated, NPPB-sensitive Cl⁻ currents (Iamil-NPPB) of permeabilized alveolar monolayers compared in nontreated and pinacidil-treated (100 μM, basolateral side, 24 h; n = 17) conditions. Mean differences and significance are also indicated.
Control of ion and fluid transport by K⁺ channels in alveolar cells

Fig. 5. Inhibition of ENaC mRNA expression by K⁺ channel inhibitors. Alveolar epithelial cells were cultured on permeant filters for 3 days and treated for an additional 24 h on the basolateral side with K⁺ channel inhibitors, glibenclamide (glib bas; a KATP inhibitor, 100 μM), clofilium (a KvLQT1 inhibitor, 5 μM), and a combination of these 3 inhibitors (3inh). The effect of apical glibenclamide was also tested (glib ap, 100 μM). To study the impact of membrane depolarization and increase in intracellular K⁺ (ValKCl), in the absence of the K⁺ inhibitor the basolateral side of the monolayers was also treated (24 h) with valinomycin (1 μM) in high-K⁺ (60 mM KCl in the culture medium). α- (A), β- (B), and γ- (C) ENaC mRNA expressions, normalized to β-actin, are represented as % of control monolayers.

Fig. 6. Inhibition of ENaC protein expression by K⁺ channel inhibitors. The relative amount of α-ENaC protein (B) was determined by Western blot analysis using an α-ENaC antibody (PA1-920, ABR) in alveolar whole cell lysates (50 μg protein) of nontreated monolayers (Ctl) or treated with basolateral glibenclamide (n = 11) and with clofilium (n = 6). A representative chemiluminescent reaction is also presented (A). Untransfected Madin-Darby canine kidney cells (MDCK -ENaC, 1 μg protein) and MDCK cells transfected with rat α-ENaC tagged with the influenza hemagglutinin epitope (MDCK +ENaC, 1 μg protein) served as negative and positive controls. The specificity of the antibody was also verified with a blocking peptide on nontreated alveolar epithelial cell lysates (CtlPep).
Effect of K⁺ channel modulators on CFTR expression. We observed that glibenclamide, applied on the apical or basolateral side of the monolayers (n = 8), as well as clofilium (n = 6) or Tram34 (n = 9) did not modify CFTR mRNA expression (Fig. 8A). However, combined treatment with basolateral glibenclamide, clofilium, and Tram34 induced a slight but significant decrease of CFTR mRNA (19.7 ± 6.6%; P = 0.04, n = 5; Fig. 8A). Finally, valinomycin and high-K⁺ treatment, in the absence of the K⁺ channel inhibitors, also reduced CFTR expression by 73.3 ± 11.1% (P = 0.004, n = 7).

Since the decrease of cAMP-stimulated Cl⁻ current in the presence of a single K⁺ channel inhibitor (glibenclamide or clofilium alone) was observed in the absence of CFTR mRNA expression inhibition, we hypothesized that posttranscriptional events could also be involved. Indeed, we found that 24-h treatment with glibenclamide severely reduced CFTR expression (59 ± 17% inhibition; n = 5, P = 0.025; Fig. 8B) measured by Western immunoblotting. Clofilium induced lower inhibition of CFTR expression (29.6 ± 7% inhibition; P = 0.022, n = 4; Fig. 8B).

Effect of pinacidil, an activator of K₅ ATP channels, on CFTR expression. CFTR mRNA expression was compared on monolayers treated or not with pinacidil (Fig. 9A). The treatment had a larger effect (46 ± 8% increase; P = 0.0004, n = 11) on CFTR expression than that observed on α-ENaC mRNA (P = 0.01). This mRNA increment was associated with a 34.1 ± 4.1% elevation of CFTR protein expression in pinacidil-treated monolayers (Fig. 9B; P < 0.0001, n = 5).

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**Fig. 7.** Stimulation of ENaC expression by pinacidil, an activator of K₅ ATP channels. Alveolar epithelial cells were cultured on permeant filters for 3 days and treated for an additional 24 h with pinacidil (100 μM). A: α-, β-, and γ-ENaC mRNA expression levels of control and pinacidil-treated monolayers, normalized to β-actin (n = 10). B: a representative chemiluminescent reaction shows increased α-ENaC protein expression. The mean relative amount of α-ENaC protein in cell lysates from pinacidil-treated monolayers was calculated as % of control monolayers (n = 7).

**Fig. 8.** Inhibition of CFTR mRNA and protein expression by K⁺ channel inhibitors. Alveolar epithelial cells were cultured on permeant filters for 3 days and treated for an additional 24 h at the basolateral side with K⁺ channel inhibitors glibenclamide (a K₅ ATP inhibitor, 100 μM), clofilium (a KvLQT1 inhibitor, 5 μM), Tram34 (an IKCa inhibitor, 5 μM) or a combination of the 3 (3inh). The impact of membrane depolarization and increase in intracellular K⁺ (ValKCl), in the absence of the K⁺ channel inhibitors, was also tested after treatment at the basolateral side of monolayers with valinomycin (1 μM) in high-K⁺ medium (addition of 60 mM KCl in the culture medium). A: CFTR mRNA expression, normalized to β-actin, is represented as % of control monolayers. B: the relative amount of CFTR protein was measured by Western blot analysis with a CFTR antibody (H182, Santa Cruz Biotechnology) in alveolar whole cell lysates (120 μg protein) of nontreated monolayers (control) or monolayers treated with glibenclamide (n = 5) or clofilium (n = 4). A representative chemiluminescent reaction is also presented.
Amiloride (10 μM, 24 h) increased the small increase of alkali cation transport 
(n = 0.0123), measured for a 24-h period in the presence or absence of KCl. 

Across Alveolar Epithelial Cell Monolayers

Impact of K⁺ Channel Modulators on Fluid Transport Across Alveolar Epithelial Cell Monolayers

Fluid transport from the apical to the basolateral side of alveolar monolayers (cultured for 4 days at the ALI) was measured for a 24-h period in the presence or absence of K⁺ channel modulators (Fig. 10). Mean fluid absorption in non-treated monolayers was 0.72 ± 0.07 μl·cm⁻²·h⁻¹ (n = 12), a value comparable to that reported recently by Fang et al. (Ref. 21; 0.84 μl·cm⁻²·h⁻¹). Basal fluid transport was reduced by 47.1 ± 5.4% (P < 0.0001, n = 10) after inhibition by amiloride (10 μM, apical side) of Na⁺ transport through ENaC HSC and NSC channels. Clofilium and glibenclamide elicited an effect similar to that of amiloride on fluid absorption, i.e., 62.5 ± 13.1% (P = 0.017, n = 4) and 38.5 ± 10.1% (P = 0.0123, n = 6) inhibition, respectively. Consistent with the small increase of Jₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ¢
longed (Figs. 2 and 3) application of clofilium, a specific inhibitor of KvLQT1 channels, confirmed the activity of KvLQT1 channels in cultured alveolar epithelial cells. The difference between our data and previously reported data (14) could be explained by a species difference or the low sensitivity of the in situ hybridization technique used.

High-conductance, charybdotoxin-sensitive KCa channels (maxi-KCa or Slo1) were previously found in the alveolar A549 cell line and in nasal cells (28, 29, 52). The intermediate-conductance KCa channel (IKCa channel), which is sensitive to clotrimazole, Tram34, and charybdotoxin, has been also reported in A459 cells as well as in the trachea and bronchi (12, 39, 58, 60). The detection of IKCa and Slo1 transcripts in alveolar cells in primary culture reported in our study is consistent with the presence of these two types of KCa channels in A549 cells. However, the presence of these proteins could not be confirmed in our immunoblot experiments. This absence of signal could be due to the low efficiency of the antibodies or to a low level of expression in control conditions. Further experiments will be needed to determine the difference in PCR and Western blotting data. Contrary to our PCR results, O’Grady and Lee (47) reported in a recent review that neither IKCa nor Slo1 mRNA was detected in alveolar epithelial cells. However, differences in culture conditions, which are known to modulate ion channel expression (10, 24, 31), could explain this discrepancy. Indeed, we observed that Kir6.1 (31) and KvLQT1 (Fig. 1) expression significantly decreased between days 0 and 4, whereas a nonsignificant increase of KCa (IKCa and Slo1) channel expression was recorded (Fig. 1). It should be noted that the freshly isolated cell mix contained ~85% alveolar cells (10). Even if minor contamination with blood cells was not excluded, we estimated that the major part of the nonalveolar cells included in the cell mix was comprised of macrophages (10). IKCa channels, in particular, are expressed in macrophages and blood cells (erythrocytes, lymphocytes, and platelets) (15, 18, 19, 33, 48, 55). Thus contamination with IKCa transcripts of macrophages and blood cells is not excluded in freshly isolated mRNA. However, these contaminating cells are completely absent in cultured alveolar cell monolayers.

The cellular localization of KATP, KvLQT1, and KCa channels has not been clearly defined in lung epithelia. Our acute (31) and chronic (present study) functional experiments suggest a major basolateral localization of KATP, KvLQT1, and KCa channels. Apical localization of these channels could not be completely excluded, but their level of expression was probably very low, since we did not detect any effect of K+ channel inhibitors at the apical membrane. Conversely, chroomanol 293B-sensitive Ica (KvLQT1 currents) have been reported (43) at the apical but not at the basolateral membrane in airway serous cells (Calu-3). Consistent with this result, the authors noted apical localization of KvLQT1 channels in immunolocalization experiments. Thus the membrane localization of K+ channels could differ in alveolar and bronchial epithelial cells.

**Effect of Long-Term Treatment with K+ Channel Modulators on Transepithelial Currents in Alveolar Epithelial Cells**

We recently reported (31) that short-term treatments with KATP, KvLQT1, or KCa inhibitors reduced Na+ and Cl− currents in alveolar cell monolayers. On the other hand, even if it has been shown (53) that a KATP opener increased K+ transport and alveolar clearance in human lungs, by a mechanism probably involving ENaC channels, a long-term effect of KATP channel activity on Na+ transport has never been directly demonstrated. We therefore decided to study the impact of long-term modulation of KATP, KvLQT1, and KCa channel activities on Na+ and Cl− transepithelial currents. We observed that 24-h treatment with glibenclamide or clotrimazole reduced basal Ica (inhibition of 60% and 68%, respectively) as well as amiloride-sensitive Na+ currents (inhibition of 74% and 75%) and forskolin-activated Cl− currents (inhibition of 41% and 44%) in intact alveolar monolayers. Although IKCa and maxi-KCa inhibitors were efficient on forskolin-activated Cl− currents, their inhibitory effects were lower than those of KvLQT1 and KATP inhibitors.

The impact of K+ channel inhibitors on the proportion of active ENaC channels at the apical membrane was then evaluated in basolaterally permeabilized alveolar monolayers. In these conditions, we found that clotrimazole, an inhibitor of KvLQT1 channels, reduced ENaC HSC currents (sensitive to 1 μM amiloride) by 56%, whereas no effect was observed on NSC currents (sensitive to 10 μM amiloride). These results confirm that K+ channel inhibitors severely decrease ENaC HSC activity and/or expression at the apical membrane.

Since KATP channel activation was shown to stimulate alveolar clearance (53), we tested, in a second step, the effect of a KATP opener on Na+ and Cl− transport. Because YM-934, used in that study (53), was not available commercially we chose pinacidil, which is commonly used to activate KATP. We observed that basal Ica as well as amiloride-sensitive currents were stimulated by 24-h treatment with pinacidil in intact alveolar monolayers. This sustained increase in Na+ transport after KATP activation could explain the stimulation of alveolar clearance (Ref. 53 and Fig. 10). Unlike short-term treatment with pinacidil, which elevated forskolin-stimulated Cl− current (31), long-term treatment failed to stimulate Cl− transport in intact monolayers (Fig. 4A). However, it should be noted in that Figure 4 that the forskolin-stimulated Cl− currents increase slowly. A very low amount of active CFTR channels at the apical membrane could explain this observation. Nevertheless, we recently demonstrated (9, 10) the presence of functional CFTR channels in alveolar cells. We then hypothesized that Cl− secretion could be rate limited by Cl− transporters at the basolateral membrane, since the activity of Na+(K+)-2Cl− cotransport is relatively weak in alveolar cells (57). To verify whether the basolateral membrane is rate limiting, we decided to permeabilize the basolateral membrane of alveolar monolayers in the presence of a basolateral-to-apical Cl− gradient. In these conditions, forskolin induced a rapid and large increase in Cl− current (Fig. 4B), confirming our hypothesis. We also found that 24-h treatment with pinacidil induced a slight but significant increment of apical Cl− current in permeabilized monolayers (Fig. 4C). This result indicated that the proportion of active CFTR channels at the apical membrane could be upregulated after activation of KATP channels. The effect of pharmacological activation of KvLQT1 and KCa channels was not tested, since there was no specific pharmacological agent activating KvLQT1 channels, and long-term treatment with 1-ethyl-2-benzimidazolone (1-EBIO), an activator of IKCa channels, induced a significant decrease in Rsa.
Impact of $K^+$ Channel Activity on ENaC and CFTR Expression

Several hypotheses could explain the coupling of $K^+$ channel activity with $Na^+$ and $Cl^-$ transport. After short-term modulation of $K^+$ channels, the changes in electrochemical gradients would predictably affect the flow of $Na^+$ and $Cl^-$ ions. However, on prolonged treatment with $K^+$ channel modulators, the observed modulation of $Na^+$ and $Cl^-$ transport could be consecutive to multiple cellular events inducing sustained changes in ion channel activities as well as the modification of a number of expressed channels. We then explored the possibility that $K^+$ channel activity could influence ENaC and/or CFTR expression at mRNA and protein levels.

We noted that inhibitors of K$_{ATP}$ or KvLQT1 channels used alone or in combination significantly reduced the mRNA expression of $\alpha$-, $\beta$-, and $\gamma$-ENaC subunits. It must be mentioned that the effects of K$_{ATP}$, KvLQT1, and IK$_{Ca}$ inhibitors were additive. In addition, the inhibitory influence of $K^+$ channel blockers on ENaC expression could be mimicked by membrane depolarization and an increase in intracellular $K^+$ concentration ([K$^+$]). In contrast, pinacidil, an activator of K$_{ATP}$ channels, enhanced ENaC expression. Since the cellular mRNA level depends on gene transcription as well as mRNA stability, it would be necessary in the future to clarify whether $K^+$ channel activity directly or indirectly modifies ENaC promoter activity or rates of mRNA degradation. K$_{ATP}$ and KvLQT1 inhibitors and activators also regulated ENaC protein expression, which is consistent with the observed changes in $Na^+$ transport.

CFTR mRNA expression was also reduced by $K^+$ channel inhibitors applied in combination or by membrane depolarization. However, K$_{ATP}$ and KvLQT1 inhibitors were ineffective when applied alone, whereas they efficiently reduced CFTR protein expression. Our results indicate that transcriptional as well as posttranscriptional regulatory mechanisms could be involved in the control of CFTR expression after changes in $K^+$ channel activities.

We also found that pinacidil stimulated CFTR mRNA and protein expression. However, as detailed below, this increase in CFTR expression was coupled to an elevation of cAMP-stimulated $Cl^-$ currents in basolaterally permeabilized monolayers only. As detailed below, we believe that such results can be explained, at least in part, by the low efficiency of basolateral $Cl^-$ transporters, such as the $Na^+$-$K^+$-$2Cl^-$ cotransporter, which probably rate limited $Cl^-$ secretion in intact monolayers.

Actually, the intracellular mechanisms responsible for both transcriptional and posttranscriptional regulation of ENaC and CFTR expression are not well defined. The aim of this study was not to determine the regulatory mechanisms of ENaC and CFTR expression by $K^+$ channel modulators, but rather the hypotheses merit exploration in the future. Different cellular signals could be implicated, including changes in membrane potential. We demonstrated, for example, that membrane depolarization after valinomycin application in the presence of high-$K^+$ medium reduced ENaC and CFTR expression. The additive inhibitory effect of $K^+$ channel blockers observed in our study also confirmed that the targets of $K^+$ channel blockers are the $K^+$ channels directly, their simultaneous action probably producing larger membrane depolarization.

However, if membrane depolarization after acute application of $K^+$ blockers is easy to explain, it is not certain whether this change in membrane potential could be sustained by chronic exposure. Nevertheless, a sustained decrease of membrane potential has already been reported in arterial smooth muscle from hypertensive animals, which exhibited reduced voltage-dependent $K^+$ current together with membrane depolarization and elevated intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) compared with normotensive rats (59).

Such changes in membrane potential could have several consequences on intracellular ion concentrations, which could control ion channel expression. For example, an increase in $[Ca^{2+}]_i$, coupled to membrane depolarization has been associated with the activation of transcription factors (41, 59). In addition, intracellular calcium has been demonstrated to regulate both CFTR (1) and ENaC (50) expression. On the other hand, it has been proposed that intracellular concentrations of monovalent ions could also act as second messengers. $[K^+]_i$, for example, has been shown to regulate protein phosphatase A2 activity, which is responsible for dephosphorylation and deactivation of CFTR in the sweat ducts (51). This cation is also involved in protein synthesis in several cell types (11, 30, 40, 49). In addition to changes in $[K^+]_i$, membrane depolarization could also be responsible for intracellular $Na^+$ or $Cl^-$ concentration changes, which have been demonstrated, for example, to regulate $\alpha$-ENaC expression (46).

Regulation of Fluid Transport by $K^+$ Channel Modulators

Since $K^+$ channel modulators affected $Na^+$ and $Cl^-$ transport, we decided to test their impact on fluid absorption across alveolar monolayers. We first observed that the basal level of fluid reabsorption across alveolar monolayers in our study (0.72 $\mu l\cdot cm^{-2}\cdot h^{-1}$) was close to that reported recently by Fang et al. (21) (0.84 $\mu l\cdot cm^{-2}\cdot h^{-1}$). In addition, we confirmed that ENaC inhibition with amiloride reduced fluid absorption by ~50%. This result is consistent with an involvement of $Na^+$ transport in fluid absorption. We also demonstrated that KvLQT1 and K$_{ATP}$ channel activity was necessary for fluid absorption, since clofibrate and glibenclamide reduced it by 62% and 39%, respectively. This finding indicated that $K^+$ channel activities, probably through their control of ENaC and/or CFTR channel activity and expression that regulate $Na^+$ and $Cl^-$ transport, also contribute to maintain fluid clearance in reabsorbing epithelia. However, an impact of changes in $K^+$ channel activity on other transport mechanisms, potentially involved in fluid transport, is not excluded. Finally, pinacidil significantly enhanced fluid absorption. It must be noted, however, that the level of stimulation of $Na^+$ and fluid transport was relatively low. It would be interesting to apply K$_{ATP}$ and KvLQT1 channel activators in combination to potentiate their activating effects. Unfortunately, KvLQT1 channels lacked specific openers.

In summary, our study demonstrates that freshly isolated and cultured alveolar cell monolayers expressed K$_{ATP}$, KvLQT1, IK$_{Ca}$, and Slo1 $K^+$ channels. We observed that long-term modulation of $K^+$ channel activities exerted sustained control in $Na^+$, $Cl^-$, and fluid transport, which involves the regulation of $\alpha$-, $\beta$-, and $\gamma$-ENaC as well as CFTR expression.
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