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

Claudie Leroy, Anik Privé, Jean-Charles Bourret, Yves Berthiaume, Pasquale Ferrari, and Emmanuelle Brochiero. Regulation of ENaC and CFTR expression with K⁺ channel modulators and effect on fluid absorption across alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 291: L1207–L1219, 2006. First published August 4, 2006; doi:10.1152/ajplung.00376.2005.—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/sulfonylurea 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 K⁺ 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 (KATP and KvLQT1 inhibitors) strongly reduced basal transepithelial current, amiloride-sensitive Na⁺ current, and forskolin-stimulated Cl⁻ currents, 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.

lungs; ATP-sensitive K⁺ channel; KvLQT1; calcium-activated K⁺ channel; Na⁺ and Cl⁻ transepithelial transport; alveolar 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 sulfonylurea 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⁺ (KCa) 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 epithelia (5), 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.

Address for reprint requests and other correspondence: E. Brochiero, Centre de recherche, CHUM-Hôpital-Dieu, 3850 St-Urbain, Montreal, QC H2W 1T7, Canada (e-mail: emmanuelle.brochiero@umontreal.ca).
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).

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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 Moloney murine leukemia virus reverse transcriptase (RT, Invitrogen) in the presence of (oligo)dT primers. cDNAs were amplified with Taq polymerase (Invitrogen), using specific primers designed from sequences of the following cloned K+ channels: rat KvLQT1 (Ref. 27, GenBank Accession No. AJ133685), Slo1 (GenBank Accession No. NM031828), IKCa (Ref. 45; GenBank Accession No. AJ133685), and Slo1 (GenBank Accession No. AJ133685), KvLQT1, Slo1, and IKCa primers, respectively, were extracted from agarose gel with QIAEXII (Qiagen, Mississauga, ON, Canada), according to the manufacturer’s instructions and purified by filtration on Microcon-PCR filters (Amicon, Beverly, MA). The nucleotide sequences of the purified products were confirmed by sequencing at the Centre hospitalier de l’Université de Montréal (CHUM) sequencing facilities with a ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The identity of ENaC and CFTR products has already been confirmed by sequencing in previous studies (10, 13).

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 extraction. 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, Santa Cruz, CA). 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 NulI (kindly provided by Dr. S. T. 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 lysis 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, pHe 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 fluoride 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, PAI-920; Affinity BioReagents, Golden, CO), anti-Kir6.1 (dilution 1:200, sc-11224, Santa Cruz Biotechnology), anti-KvLQT1 (dilution 1:500, 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).
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 g of protein) and transfected MDCK cells with rat α-ENaC tagged with the influenza hemagglutinin epitope (1 g 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 voltohmeter (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 (Rte, Ω·cm²) across the cell monolayers. Transepithelial current (Isc) across the monolayers was then calculated according to the following formula: $I_{sc} = PD/R_{te}$ (13). To quantify the amount of amiloride-sensitive current generated by intact alveolar monolayers treated for 24 h with or without K⁺ channel inhibitors, $I_{sc}$ 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 $I_{sc}$ 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 0 mV with either two Ag-AgCl electrodes, and the resulting $I_{sc}$ 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, $I_{sc}$ 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 μCi/ml 125I-albumin was added to the apical side of the monolayers. The differentiation and permeability of MDCK-IAG cells were then incubated at 37°C with 5% CO₂ in an humidified incubator. After 5 min ($t_b$), 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 ($t_s$). The $t_b$ and $t_s$ 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 $t_s$ sample/weight of $t_s$ sample)/(cpm of $t_b$ sample/weight of $t_b$ sample) × 100, where cpm is counts per 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 ($P_p$) was also estimated in each monolayer. The remaining apical and basolateral media were collected; the monolayers were washed with H₂O, and the washing medium was collected and counted. $P_p$ of each monolayer was calculated according to the following formula: $P_p$ = (cpm of basolateral compartment)/(cpm of apical compartment), where cpm of the apical compartment was cpm of the $t_b$ sample + cpm of the $t_s$ sample + cpm of the remaining apical volume + cpm of the apical washing solution. Mean $P_p$ was 0.57 ± 0.05%; monolayers with $P_p$ >3% were discarded. Free 125I measured in the presence of trichloroacetic acid (20%) was <2.5%. Evaporative loss of fluid measured in an empty, Paraffilm-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 purified. Sequencing of the purified products confirmed 100% identity to cloned rat IKCa (GenBank Accession no. NM023021), rat Slo1 (maxi-KCa, GenBank Accession no. NM031828), and rat KvLQT1 (GenBank Accession no. AJ133685). 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.
Since we recently found (31) that expression of the Kir6.1 and SUR2B subunits (forming the alveolar K_{ATP} 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 IK_{Ca}, 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 IK_{Ca} and Slo1 expression between days 0 and 4.

Expression of KvLQT1, Kir6.1, IK_{Ca}, 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-IK_{Ca} antibody detected several bands, one of them at ~55 kDa, the expected mass of IK_{Ca} (data not shown). However, the specificity of this band could not be verified because the blocking peptide was unavailable. Finally, an anti-rabbit maxi-IK_{Ca} (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

We have already shown (31) that short-term treatment with K_{ATP}, KvLQT1, and K_{Ca} 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 I_{Ce} was 5.0 ± 0.1 µA/cm^2 (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 K_{ATP}; Fig. 2A; n = 14) and clotrimazole (20 µM), an inhibitor of K_{Ca} channels, also significantly decreased basal I_{Ce} (25% inhibition, P < 0.0001; n = 18). Other inhibitors of K_{Ca} channels, charybotoxin (200 nM; an inhibitor of maxi-K_{Ca} and IK_{Ca} channels), Tram34 (5 µM; an inhibitor of IK_{Ca} channels), and iberiotoxin (100 nM; an inhibitor of maxi-K_{Ca} channels), elicited nonsignificant declines of basal I_{Ce}.

Treatment with K^{+} channel inhibitors also influenced amiloride-sensitive Na^{+} currents. In control monolayers, 65 ± 1% of basal I_{Ce} was sensitive to amiloride (I_{Ce-amil} of 3.2 ± 0.1
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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 KᵦT₅ channels, on alveolar transepithelial currents. Since it has been shown that prolonged activation of KᵦT₅ 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 Iₑ 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, Iₑ decreased 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% increase (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 Iₑ (an increment of 10.3 ± 1.4 μA/cm² after 10 min; n = 17; Fig. 4B) in permeabilized monolayers compared with intact monolayers.
This cAMP-stimulated and NPPB-sensitive Cl\(^{-}\) current was significantly enhanced by pinacidil treatment (mean difference of 2.6 \(\mu\)A/cm\(^2\) 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 \(\mu\)M, basolateral side) reduced \(\alpha\)-ENaC expression by 29 \(\pm\) 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 \(\alpha\)-ENaC expression (increase of 1.5 \(\pm\) 10.9%; NS.)
nontreated monolayers (Fig. 7 and 10) expression in pinacidil-treated monolayers compared with blotting (18.1 -ENaC protein expression was apparent on Western immunoblotting, was reduced by 52.7 and 27.2 %). The level of -ENaC protein, estimated by Western immunoblotting, was reduced by 52.7 % (P = 0.0053, n = 4) and 27.2 % (P = 0.029, n = 6) after KATP and KvLQT1 channel inhibition by glibenclamide and clofilium, respectively (Fig. 6).

The level of -ENaC protein, estimated by Western immunoblotting, was reduced by 52.7 % (P = 0.0053, n = 4) and 27.2 % (P = 0.029, n = 6) after KATP and KvLQT1 channel inhibition by glibenclamide and clofilium, respectively (Fig. 6).

Consistent with the 23% stimulation of amiloride-sensitive currents by pinacidil, semiquantitative RT-PCR experiments revealed a slight but significant increase of -ENaC (17 ± 5%; P = 0.008, n = 11) and -ENaC (21.7 ± 9.1%; P = 0.04, n = 10) expression in pinacidil-treated monolayers compared with nontreated monolayers (Fig. 7A). Also, a 32% nonsignificant rise of -ENaC expression was recorded. A similar increase of -ENaC protein expression was apparent on Western immunoblotting (18.1 ± 7.1% increment in pinacidil-treated monolayers; P = 0.04, n = 7; Fig. 7B).

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), Tram34 (an IKCa inhibitor, 5 μM), or 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+ medium (addition of 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 -ENaC protein (B) was determined by Western blot analysis using an -ENaC antibody (PA1-920, ABR) in alveolar whole cell lysates (50 μg protein) of nontreated monolayers (Ctl) or treated with basolateral glibenclamide (n = 4) or 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 -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 (CilPep).
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 inhibitor, 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 K_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).

Fig. 7. Stimulation of ENaC expression by pinacidil, an activator of K_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 K_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⁺ 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 = 7) or clofilium (n = 4). A representative chemiluminescent reaction is also presented.
amiloride (10 μM, apical side) of Na\(^+\) transport through ENaC and Cl\(^-\) 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 \(I_{\text{amil}}\) by pinacidil, this activator heightened fluid absorption by 21.9 ± 8.3% (P = 0.0331, n = 8).

**DISCUSSION**

The results of this study demonstrate the presence of KvLQT1 transcript as well as \(I_{\text{KCa}}\) and Slo1 transcripts coding for KvLQT1, \(I_{\text{KCa}}\), and maxi-K\(_{\text{Ca}}\) channels, respectively, in alveolar epithelial cells. More interestingly, we demonstrated that long-term treatment with modulators of KvLQT1 and \(K_{\text{ATP}}\) channels affected ENaC and CFTR expression (at the mRNA and protein levels). The effect of K\(^+\) channel modulators could be mimicked by membrane depolarization, suggesting that changes in membrane potential could be involved in the regulation of ENaC and CFTR expression. Finally, the impact of K\(^+\) channel activities on fluid absorption in alveolar epithelia could be explained, at least in part, by a modulation of Na\(^+\) and Cl\(^-\) transport.

**Molecular Identity and Expression of K\(^+\) Channels**

In a previous study (31), we identified the molecular identity of the subunits (Kir6.1 and SUR2B) forming \(K_{\text{ATP}}\) channels in alveolar epithelial cells. A similar RT-PCR strategy allowed us to identify KvLQT1, \(I_{\text{KCa}}\), and Slo1 (maxi K\(_{\text{Ca}}\)) transcripts. Immunoblotting experiments confirmed the presence of Kir6.1 and KvLQT1 proteins in alveolar monolayers. A band corresponding to the molecular mass of \(I_{\text{KCa}}\) was also detected, but its specificity could not be confirmed. Conversely, we were not able to detect a signal for Slo1 (maxi-K\(_{\text{Ca}}\)) proteins.

To date, the presence of KvLQT1 and \(I_{\text{KCa}}\) channels has not been clearly demonstrated in alveolar epithelial cells. Indeed, KvLQT1 channels have been reported along airways, in nasal (34, 36), tracheal (23), and bronchial (12, 14) cells; however, a KvLQT1 antisense probe failed to stain alveoli in the mouse lung (14). Our results could not confirm this observation. We not only observed KvLQT1 by PCR but also detected a significant signal by Western blotting with 40 μg of total protein extract, which is relatively low compared with the amount necessary to detect \(I_{\text{KCa}}\) or CFTR, for example (120 μg). Furthermore, our functional experiments showing the sensitivity of transepithelial currents to short (31) and prog-
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 $K_{Ca}$ channels (maxi-$K_{Ca}$ or Slo1) were previously found in the alveolar A549 cell line and in nasal cells (28, 29, 52). The intermediate-conductance $K_{Ca}$ channel (IK$_{Ca}$ 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 IK$_{Ca}$ and Slo1 transcripts in alveolar cells in primary culture reported in our study is consistent with the presence of these two types of $K_{Ca}$ 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 IK$_{Ca}$ 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 $K_{Ca}$ (IK$_{Ca}$ 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). IK$_{Ca}$ channels, in particular, are expressed in macrophages and blood cells (erythrocytes, lymphocytes, and platelets) (15, 18, 19, 33, 48, 55). Thus contamination with IK$_{Ca}$ 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 K$_{ATP}$, KvLQT1, and K$_{Ca}$ channels has not been clearly defined in lung epithelia. Our acute (31) and chronic (present study) functional experiments suggest a major basolateral localization of K$_{ATP}$, KvLQT1, and K$_{Ca}$ 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, chromanol 293B-sensitive $I_{sc}$ (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 K$_{ATP}$, KvLQT1, or K$_{Ca}$ inhibitors reduced Na$^+$ and Cl$^-$ currents in alveolar cell monolayers. On the other hand, even if it has been shown (53) that a K$_{ATP}$ opener increased K$^+$ transport and alveolar clearance in human lungs, by a mechanism probably involving ENaC channels, a long-term effect of K$_{ATP}$ channel activity on Na$^+$ transport has never been directly demonstrated. We therefore decided to study the impact of long-term modulation of K$_{ATP}$, KvLQT1, and K$_{Ca}$ channel activities on Na$^+$ and Cl$^-$ transepithelial currents. We observed that 24-h treatment with glibenclamide or clofilium reduced basal $I_{sc}$ (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 IK$_{Ca}$ and maxi-K$_{Ca}$ inhibitors were efficient on forskolin-activated Cl$^-$ currents, their inhibitory effects were lower than those of KvLQT1 and K$_{ATP}$ 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 clofilium, 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 K$_{ATP}$ channel activation was shown to stimulate alveolar clearance (53), we tested, in a second step, the effect of a K$_{ATP}$ 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 $I_{sc}$ 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 K$_{ATP}$ 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 K$_{ATP}$ channels. The effect of pharmacological activation of KvLQT1 and K$_{Ca}$ channels was not tested, since there was no specific pharmacological agent activating KvLQT1 channels, and long-term treatment with 1-ethyl-2-benzimidazolyl (1-EBIO), an activator of IK$_{Ca}$ channels, induced a significant decrease in $R_{sc}$. 

AJP-Lung Cell Mol Physiol • VOL 291 • DECEMBER 2006 • www.ajplung.org
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 KATP or KvLQT1 channels used alone or in combination significantly reduced the mRNA expression of α-, β-, and γ-ENaC subunits. It must be mentioned that the effects of KATP, KvLQT1, and IKCa 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 KATP 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. KATP 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, KATP 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 several 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 Ca²⁺ concentration ([Ca²⁺]), 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 [Ca²⁺], 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⁺], 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⁺], membrane depolarization could also be responsible for intracellular Na⁺ or Cl⁻ concentration changes, which have been demonstrated, for example, to regulate α-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 ± 0.20 μl·cm⁻²·h⁻¹) was close to that reported recently by Fang et al. (21) (0.84 μl·cm⁻²·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 KATP 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 KATP 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 KATP, KvLQT1, IKCa, 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 α-, β-, and γ-ENaC as well as CFTR expression.
ACKNOWLEDGMENTS

We thank Dr. M.A. Matthay for his protocol and kind advice on the fluid transport experiments. We also acknowledge the editorial assistance of Ovid Da Silva, Research Support Office, Research Centre, CHUM.

GRANTS

This work was supported by the CHUM Research Centre Foundation and the Natural Sciences and Engineering Research Council of Canada (Grant 312177-05). E. Brochiiero was the recipient of a scholarship from Fonds de la recherche en santé du Québec (FRSQ).

REFERENCES


