Evidence of a functional CFTR Cl\(^{-}\) channel in adult alveolar epithelial cells

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Brochiero, Emmanuelle, André Dagenais, Anik Privé, Yves Berthiaume, and Ryszard Grygorczyk. Evidence of a functional CFTR Cl\(^{-}\) channel in adult alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 287: L382–L392, 2004.—The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in the fetal lung, but during lung development it gradually disappears in cells of future alveolar spaces. Recent studies have implicated the CFTR in fluid transport by the adult alveolar epithelium, but its presence has not been demonstrated directly. This study re-evaluated CFTR expression and activity in the adult pulmonary epithelium by using freshly isolated rat alveolar type II (ATII) cells. CFTR mRNA was detected by semiquantitative polymerase chain reaction on the day of cell isolation but was rapidly reduced by 60% after 24 h of cell culture. This was paralleled by a similar decrease of surfactant protein A expression and alkaline phosphatase staining, markers of the ATII cell phenotype. CFTR expression increased significantly on day 4 in cells grown on filters at the air-liquid interface compared with cells submerged or grown on plastic. Significantly higher CFTR expression was detected in distal lung tissue compared with the trachea. The CFTR was also found at the protein level in Western blot experiments employing lysates of freshly isolated alveolar cells. Whole cell patch-clamp experiments revealed cAMP-stimulated, 5-nitro-2-(3-phenylpropylamino)benzoate-sensitive Cl\(^{-}\) conductance with a linear current-voltage relationship. In cell-attached membrane patches with 100 μM amiloride in pipette solution, forskolin stimulated channels of \(\sim\)4 pS conductance. Our results indicate that 50–250 of functional CFTR Cl\(^{-}\) channels occur in adult alveolar cells and could contribute to alveolar liquid homeostasis.

LUNG LIQUID TRANSPORT IS ESSENTIAL for development of the fetal lung, the maintenance of a thin fluid layer in adult lung alveoli, and the resolution of pathological conditions, such as pulmonary edema. It is osmotically driven by active movement of solutes, mainly Na\(^{+}\) and Cl\(^{-}\), across the pulmonary epithelium (for review, see Refs. 29, 31, 32). In the fluid-filled fetal lung, Cl\(^{-}\)-driven secretion is a predominant process (33, 34, 50). However, in the adult lung, effective gas exchange requires only a thin fluid layer along the apical surface of the alveolus. The removal of alveolar fluid at birth and its efficient control in the adult lung require a shift in the balance between fluid secretion and absorption processes. This transition is associated with increased, active Na\(^{+}\) absorption and reduced Cl\(^{-}\) secretion across the alveolar epithelium. Adult lung alveolar type II (ATII) cells are thought to control the composition and volume of alveolar fluid (28, 31). However, recent findings show that alveolar type I (ATI) cells, which cover \(\sim\)95% of the alveoli surface, may also contribute to lung liquid homeostasis (24, 43).

Transepithelial Na\(^{+}\) absorption involves passive entry into alveolar cells via amiloride-sensitive apical Na\(^{+}\) channels, mainly epithelial Na\(^{+}\) channels (ENaC) (5, 13, 30, 36). Na\(^{+}\) ions are subsequently extruded through basolateral Na/K-ATPase (for review, see Refs. 28, 29). Liquid secretion consists of Cl\(^{-}\) entry through a basolateral Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\)cotransporter, followed by its exit via apical Cl\(^{-}\) channels, such as the cystic fibrosis transmembrane conductance regulator (CFTR). In situ hybridization and immunolocalization experiments have revealed that the CFTR is present in fetal alveolar cells, where its contribution to Cl\(^{-}\) secretion is well documented (27, 33, 50). However, CFTR expression diminishes in future alveolar spaces during lung development and remains confined to bronchiolar epithelia (35, 38, 50). In situ hybridization as well as immunohistochemistry studies have not always disclosed the CFTR in adult alveolar cells. Some investigations did not detect the CFTR (38, 50), whereas others found low expression levels (11, 44). Similarly, some functional studies reported cAMP-activated Cl\(^{-}\) transport across ATII cells cultured from rabbits (39) and rats (22). However, patch-clamp experiments either gave no evidence of CFTR channels in adult rat ATII cells (53), or, when Cl\(^{-}\) channels were noted, their properties were not entirely consistent with those expected for the CFTR (22). The presence of the CFTR in the distal lung epithelium might not be surprising, considering its possible involvement in lung fluid homeostasis. Recent studies indicate that CFTR-mediated Cl\(^{-}\) transport, in addition to its participation in fluid secretion, may also play a role in cAMP (or terbutaline)-stimulated Na\(^{+}\) absorption by ATII cells (26, 41) and in cAMP stimulation of lung liquid clearance in vivo (12). However, direct evidence of functional CFTR channels in adult ATII cells is lacking.

Semiquantitative RT-PCR was performed in this study to detect CFTR cDNA in adult rat alveolar cells, trachea, and distal lung. The level of CFTR expression in alveolar cells diminished rapidly during cell culture and was dependent on the culture conditions. For this reason, Western blot and patch-clamp experiments were performed on freshly isolated cells to improve the detection of CFTR channel protein. In whole cell and cell-attached patch-clamp experiments, we found cAMP-activated currents with biophysical and pharmacological properties consistent with CFTR Cl\(^{-}\) channels. Our work provides direct evidence of functional CFTR channels in the adult alveolar epithelium.

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MATERIALS AND METHODS

Alveolar Epithelial Cell Isolation and Primary Culture

ATII cells were isolated from male Sprague-Dawley rats, following the protocol described previously (14). Briefly, after anesthesia, tracheotomy, and cannulation of the pulmonary artery, the lungs were perfused and inflated simultaneously with air. After removal from the thoracic cavity, they were washed 10 times and digested enzymatically with elastase (Worthington Biochemical, Freehold, NJ). The lungs were then minced, and the resulting suspension was filtered. Alveolar cells were purified by a differential adherence technique, which enhances the purity of the ATII cell pool (6). Briefly, “pre-IgG” cell suspensions were incubated for 45–60 min on bacteriological plastic plates coated with rat IgG at 37°C in a 5% CO2 incubator. Most of the macrophages were then bound to IgG, whereas nonadherent “post-IgG” cells, which contained ~85% of ATII cells (see RESULTS, Fig. 2), were collected and recovered by centrifugation. They were resuspended in minimum essential medium (MEM; GIBCO, Burlington, Ontario, Canada) containing 10% FBS, 0.08 mg/l gentamicin, septrin (3 μg/ml trimethoprim + 17 μg/ml sulfamethoxazole), 0.2% NaHCO3, 10 mM HEPES, and 2 mM L-glutamine. For primary culture, the cells were plated at 1 × 106 cells/cm2 density on 4-cm2 permeable filters (Costar Transwell, Toronto, Ontario, Canada), and cultured at 37°C with 5% CO2 in an humidified incubator. The medium was replaced after 3 days by the same MEM but without septrin. To culture cells at the air-liquid interface (ALI), we collected the liquid on the apical side 24 h after cell seeding. Liquid removal was repeated every 24 h until the cells formed a tight monolayer. To study CFTR mRNA expression in cells grown on plastic, we seeded the cells in T25 Corning flasks at 4 × 105 cells/cm2.

Alkaline Phosphatase Histochemo Staining

Alkaline phosphatase staining served to identify epithelial ATII cells (10) in cytopsin preparations of freshly isolated cells as well as primary cultured cells grown on glass coverslips according to the method of Beckstead et al. (2), as described previously (14). Briefly, the cells were fixed for 30 s at room temperature in cold paraformaldehyde-acetone solution (4% wt/vol paraformaldehyde, 0.02% wt/vol Na2HPO4, 0.1% wt/vol KH2PO4, and 45% vol/vol acetone, pH 6.6), rinsed for 1 min in distilled water, and air-dried. Fixed cells were incubated for 90 min at 37°C in a fresh staining solution containing 0.05% Fast Blue BB salt in stock staining solution (30 mg of naphthol-AS-phosphate in 0.5 ml of N,N-dimethylformamide filled up to 100 ml with 0.2 M Tris base). The samples were then rinsed with distilled water for 1 min and air-dried. The cells were counterstained with 0.1% neutral red solution for 5 min at room temperature, rinsed for 1 min, air-dried, and photographed with a Nikon camera under light microscopy. Blue staining identified cells with high alkaline phosphatase activity, whereas other cells, such as macrophages, were counterstained in red.

RNA Purification

CFTR mRNA expression was analyzed in freshly isolated ATII cells (day 0) and in cells cultured for 1–4 days on permeable filters or on plastic. Total RNA was extracted by TRizol reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). CFTR mRNA expression was also tested in tracheal and distal lung tissue. For that purpose, the lungs were taken out, and the trachea and the very tip of the lungs were dissected and then homogenized immediately for 3 × 20 s with Ultra Turrax (JKA, Staufen, Germany) before RNA purification as above. The lung fragments were taken at the periphery to avoid, as much as possible, the presence of small bronchi.

Semiquantitative RT-PCR

We reverse-transcribed purified total RNA (5 μg) into single-stranded DNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of oligo dT as primer. cDNA made from 375 ng of RNA was amplified with Taq polymerase (Invitrogen) by specific primers designed for the rat CFTR (15), surfactant protein A (SP-A) (45), and β-actin (40). The PCR primer pair for the CFTR (sense: 5′-AGGAGACTGTCCCTGGTTCC-3′, exon 13, and antisense: 5′-TGGCAATTTGTAGGCTTGT-3′, exon 15; 1 μM final concentration of each) was designed to give a 499-bp specific product. The SP-A primer pair (sense: 5′-GGGATCAC-CAAGGGAGACCTGGAGAAAG-3′, exon 3, and antisense: 5′-CCGAGCTCTACAGTTGTTAGTTCAC-3′, exon 5; 1 μM final concentration of each) amplified a 371-bp product, whereas the β-actin primer pair (sense: 5′-GCTCTATCCTGGGTCATCGT-3′ and antisense: 5′-AAGCTTGGAAGTGTTGTC-3′, 0.25 μM final concentration of each) amplified a 311-bp product. To remain in the linear phase of amplification (see Fig. 1D), SP-A and CFTR products from isolated ATII cells were amplified for 25 PCR cycles, whereas β-actin amplification was stopped after 20 cycles. In addition, rTL0o, a marker for the AT1 cell phenotype, was amplified using freshly isolated cells or cells grown for 4 days under different culture conditions. The rTL0o primer pair (sense: 5′-CCAGTTGTGTCTT-GGGTTT-3′, exon 2, and antisense: 5′-GCTTTTTAGGG- GAGAAACC-3′, exon 5/6; 1 μM final concentration of each) amplified a 493-bp product. The rTL0o product was amplified for 20 cycles to remain in the linear phase of amplification. PCR products from the trachea and distal lungs were amplified on cDNA made from 500 ng of RNA. In addition to the CFTR and β-actin, cDNAs coding for keratins 18 and 19, two specific markers for epithelial cells, were also amplified. The keratin 18 primer pair (sense: 5′-AAATCCTGGA-AATCTTCTGG-3′, exon 1, and antisense: 5′-TGTCCTTCTCTC-TCTCTGAG-3′, exon 4; 1 μM final concentration of each) amplified a 471-bp product, whereas the keratin 19 primer pair (sense: 5′-GAAGATCACCAGTCGAGAC-3′ and antisense: 5′-GAATCC-TCTCCACACTGAC-3′, exon 4; 1 μM final concentration of each) amplified a 477-bp product. To remain in the linear phase of amplification, CFTR products from the trachea and distal lung were amplified for 28 PCR cycles, and keratins 18 and 19 for 21 cycles, whereas β-actin amplification was stopped after 18 cycles. The amplification products were separated on agarose gels stained with ethidium bromide and analyzed by Typhoon Gel Imager. For the semiquantitative evaluation of CFTR and SP-A cDNA in alveolar epithelial cells, the respective signals were normalized to β-actin and keratin 18 signals of the same sample. For quantification of the CFTR from the trachea and distal lung, each sample was normalized with respect to keratin 18, the only marker expressed similarly in both tissues. The identity of the CFTR cDNA fragment amplified by RT-PCR was confirmed by sequencing the purified RT-PCR product at the Centre hospitalier de l’Université de Montréal (CHUM) sequencing facilities with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster, CA), employing the CFTR oligonucleotides as primers.

Immunoblotting

Protein extracts from BHK cells stably transfected with empty pNUT vector lacking CFTR cDNA (negative control) and BHK cells stably transfected with human wt-CFTR (positive control) were kindly provided by Dr. J. Hanrahan, McGill University. The normal human airway epithelial cell line Nul1 (1) (a gift from Dr. J. Zabner, University of Iowa) endogenously expressing the CFTR also was tested as a positive control (52). Total protein extracts of epithelial alveolar cells from six different rats were prepared as follows: 20 × 106 freshly isolated epithelial alveolar cells were washed twice with PBS. The cell pellets were resuspended in solubilizing RIPA buffer (20 μM Tris-HCl, 1% Triton X-100, 0.08% deoxycholic acid, 1% sodium dodecyl sulfate), and frozen at −80°C. Cellular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and reacted with the following antibodies: polyclonal antibodies raised against CFTR, β-actin, and keratin 18.
0.1% SDS, 150 mM NaCl with protease cocktail (Complete Mini EDTA-free protease inhibitor cocktail; Roche, Mannheim, Germany), incubated at 4°C for 30 min, and centrifuged at 12,000 g for 5 min. The supernatant was collected, and protein content was estimated by Coomassie blue assay (Bradford; Pierce, Rockford, IL) with BSA as standard. Proteins (20 μg from BHK, 50 μg from NuLi, and 120 μg from ATII cells) were solubilized in sample buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 125 mM DTT), separated by 6.5% polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes that were blocked with 5% milk in Tris-buffered saline (TBS)-Tween (500 mM NaCl, 20 mM Tris-HCl, and 0.1% Tween 20, pH 7.4) for 1 hr at room temperature and then stained with primary antibody, anti-CFTR (1:500, N-Term, H-182; Santa Cruz Biotechnology, Santa Cruz, CA), in TBS plus 1% milk, for 16 h at 4°C. The membranes were washed three times in TBS-Tween for 15 min and once in TBS for 15 min and then incubated with the secondary antibody, anti-rabbit IgG linked to horseradish peroxidase (1:4,000; Cell Signaling Technology, Beverly, MA) in TBS plus 1% milk for 1 hr at room temperature. They were washed three times in TBS-Tween for 15 min and once in TBS for 15 min and developed with chemiluminescent reagent (ECL; Amersham, Buckinghamshire, UK) for 1 min. Finally, a diagnostic film (Kodak, Rochester, NY) was exposed with the membranes for 3 min.

**Electrophysiology**

**Patch-clamp recordings.** The standard patch-clamp technique (18) was employed to record whole cell and single channel currents. Patch pipettes were pulled in two stages (Narishige PP-82 puller) from borosilicate glass capillaries (catalog no. PG52151; World Precision Instruments, Sarasota, FL) and had a resistance of 2–5 megaohm when filled with 100 mM CsCl pipette solution. Ion currents were recorded with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA), sampled at 2 kHz, and stored on computer hard disk with a Digidata 1200 interface and a pCLAMP software suite (version 8, Axon Instruments). After forming whole cell configuration, membrane capacitance, membrane resistance, and access resistance were determined and checked frequently throughout the experiment to ensure proper recording of whole cell currents. The experiments were performed at room temperature.

**Cells.** In the patch-clamp experiments, freshly isolated ATII cells were kept on ice until they were used, typically within 2–6 h after isolation. During the experiments, small aliquots of cell suspension were transferred to an experimental chamber placed on the stage of an inverted microscope (Nikon Eclipse, TE300; Nikon Canada, Montreal, QC, Canada), and gigaohm seals were formed. Some control experiments were performed on macrophages. In these experiments,
IgG-coated bacteriological plates with adherent macrophages obtained during the ATII cell purification step were rinsed twice and filled with 5 ml of CsCl bath solution. The plates were mounted directly on the stage of the microscope for patch clamping. After exposure to forskolin during whole cell patch-clamp recording, the plates were discarded and fresh ones studied in subsequent experiments.

**Solutions.** The whole cell experiments were aimed at detecting Cl⁻ conductance; therefore, CsCl solutions were used in the bath and pipette to minimize currents through Na⁺ and K⁺ channels. The bath solution for the whole cell experiments contained (in mM) 145 CsCl, 1 MgCl₂, 1 CaCl₂, 10 TES, and 10 glucose, pH 7.4, adjusted with Tris base. The pipette solution contained (in mM) 100 CsCl, 2 MgCl₂, 1 EGTA, 2 Mg-ATP, and 90 sorbitol, pH 7.4. For cation-to-anion selectivity measurements, CsCl concentration in the pipette solution was lowered to 30 mM by isosmotic replacement with sorbitol. The bath and pipette solutions in the cell-attached experiments were the same as those used in the whole cell experiments, except that in the pipette solution, ATP was omitted and 100 μM amiloride was added to block Na⁺ channel currents.

**Drugs.** cAMP-elevating cocktail contained 3–10 μM forskolin, and, in some experiments, 30 μM IBMX was included. 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 500 mM stock (Sigma-Aldrich Canada, Oakville, Ontario, Canada), was prepared in DMSO.

**RESULTS**

**CFTR cDNA is Detected in Cultured and Freshly Isolated Alveolar Cells**

CFTR mRNA expression was evaluated by semiquantitative RT-PCR with RNA extracted from freshly isolated cells (day 0), as well as from cells cultured on permeable filters for 1–4 days. It has been shown previously that ATII cells cultured on plastic lose their functional differentiation, including surfactant expression (46). For this reason, SP-A mRNA expression was tested as a marker of the ATII cell phenotype, since SP-A is expressed and secreted exclusively by these cells (7). Finally, β-actin expression served to normalize the estimated CFTR and SP-A signals. The PCR primers were designed to amplify fragments of 499, 371, and 311 bp, respectively, for CFTR, SP-A, and β-actin cDNA.

Figure 1A depicts a representative agarose gel profile for the different RT-PCR products. CFTR cDNA was detected in freshly isolated ATII cells (day 0), but the levels decreased rapidly by 57.6 ± 4.3% between days 0 and 1. The expression level remained relatively stable thereafter with a 69 ± 5% total decrease between days 0 and 4 (Fig. 1, A and B). SP-A expression also declined during cell culture (Fig. 1, A and C). The 63.5 ± 6.7% SP-A reduction observed between days 0 and 4 was comparable to the estimated decrease of CFTR expression.

Alkaline phosphatase activity, another marker of the ATII cell phenotype (10), served to evaluate ATII cell content in the freshly isolated cell mix. ATII cells were identified by blue staining in alkaline phosphatase histochemical assay (Fig. 2). The differential adherence technique with rat IgG (see MATERIALS AND METHODS) significantly enhanced the proportion of ATII cells in the freshly isolated cell mix from 69 ± 3 to 86 ± 2% (n = 3) (compare the pre-IgG cell mix, Fig. 2A, and the...
post-IgG cell mix, Fig. 2C). Although most of the macrophages were bound on IgG-coated plates (Fig. 2B), some still remained in the final post-IgG cell mix, where they likely constituted a significant portion of non-ATII, red-stained cells (Fig. 2C).

Interestingly, as with CFTR and SP-A expression, alkaline phosphatase staining also decreased during primary cell culture (Fig. 2D).

**Culture Conditions Modulate CFTR Expression in Alveolar Epithelial Cells**

ATII cells are known to undergo a phenotypic change in culture and progressively lose several specific markers, such as SP synthesis (47). Cell culture at the ALI has been shown to greatly improve maintenance of the ATII cell phenotype (8). It also increases the presence of active ENaC at the cell surface (21). Because CFTR maturation and trafficking were greatly increased in Madin-Darby canine kidney (MDCK) cell monolayers grown at the ALI (1), we tested whether this approach would heighten CFTR mRNA expression in alveolar epithelial cells. We found that cells grown on filters at the ALI expressed twice as much CFTR mRNA on day 4 than cells submerged or grown on plastic ($P < 0.05$, Fig. 3). This level of CFTR mRNA was, however, still ~30% lower compared with freshly isolated cells ($P < 0.05$, Fig. 3). The levels of SP-A transcript, an ATII cell marker, or rTI 40 , an ATI cell marker, were not affected significantly at day 4 by the three culture conditions tested.

**Detection of CFTR Transcript in the Rat Trachea and Distal Lung**

CFTR transcript expression was analyzed by RT-PCR in the trachea and distal lung. Keratins 18 and 19, both markers of epithelial cells, and β-actin were also investigated. Except for keratin 18, there was a significant difference in the expression of CFTR, keratin 19, and β-actin transcripts in the trachea compared with the distal lung (Fig. 4). The CFTR signal was 26-fold stronger in the distal lung than in the trachea ($P < 0.05$, Fig. 4) when normalized relative to keratin 18 expression.

**Detection of CFTR Protein in Alveolar Epithelial Cells**

With rabbit polyclonal antibody raised against the amino terminus of CFTR (H-182, Santa Cruz Biotechnology), we then studied the presence of CFTR protein in freshly isolated alveolar epithelial cells. Positive and negative controls were deployed to test antibody specificity. A 175-kDa band, the expected molecular mass of human CFTR, was detected (Fig. 5) both in BHK cells transfected with CFTR (lane 3) and in NuLi cells (lane 1) (52). No band could be seen with BHK cells stably transfected with empty pNUT vector lacking CFTR cDNA (Fig. 5, lane 2). In alveolar epithelial cell extracts (120 μg of protein), isolated from six different rats, we observed a band at 164 kDa, which is consistent with the mature rat CFTR (150–165 kDa) (37). A lower 144-kDa band that could correspond to the unglycosylated form of CFTR was also detected. As in the four representative extracts (ATII #1–#4; Fig. 5,
solutions, formation of whole cell configuration, a time required for the pipette solution to diffuse into the cell. With CsCl solution in the bath and pipette, mean cAMP-stimulated current at the peak of the response was 101 ± 34 pA/cell (or 26.6 ± 8.9 pA/pF; n = 12, V_{rest} = −100 mV). This current was inhibited ~50% by the Cl⁻ channel blocker NPPB (100 μM, n = 6), whereas DIDS (250 μM, n = 4) was without effect, as might be expected for CFTR-mediated current (Fig. 6B, inset). This cAMP-stimulated whole cell conductance had a linear current-voltage (I-V) relationship and showed no time dependence during the 900-ms voltage steps. Its reversal potential (E_r) was shifted by approximately −20.8 ± 3.8 mV (n = 3) when Cl⁻ concentration in the pipette solution was reduced from 104 to 34 mM (Fig. 6C). This E_r shift was close to that expected from the Nernst equation (ΔE_r = −28 mV), and the difference may have arisen from several factors, including a small contribution of cation nonelective currents to the observed whole cell conductance. Thus our data are consistent with Cl⁻-selective conductance, with properties resembling those of CFTR Cl⁻ channels.

cAMP-Activated Whole Cell Cl⁻ Conductance is Absent in Macrophages

In our freshly isolated cell mix, macrophages likely constituted a significant contamination in ~15% of the non-ATII cell fraction. They could be easily distinguished from other cells by their approximately twofold larger diameter. Although such large cells were avoided during patch-clamp studies of ATII cells, smaller macrophages could have been unintentionally included in some experiments. To verify whether part of the cAMP-induced whole cell responses described above may have originated from macrophages, we performed experiments with macrophages obtained after the IgG-separation step (see MATERIALS AND METHODS). Under the same experimental conditions and solutions as with ATII cell fractions, we did not observe a response to forskolin stimulation in five whole cell recordings with macrophages (0 out of 5 cells from 2 rats, data not shown).

cAMP Activates Low-Conductance Single Channel Currents in ATII Cells

To identify Cl⁻ channels underlying cAMP-activated whole cell conductance seen in freshly isolated ATII cells, we per-

![Fig. 4](image_url)

**Fig. 4.** A: RT-PCR detection of CFTR, keratin 18, keratin 19, and β-actin in rat trachea and distal lung. The CFTR signal was normalized to keratin 18 since keratin 19 and β-actin were not expressed similarly in both tissues. B: the results are expressed as means ± SE (n = 4). K18, keratin 18. *P < 0.05.

lanes 4–7), obtained from different rats, the quantity of CFTR protein was variable from animal to animal.

cAMP Activates Whole Cell Cl⁻ Conductance in Freshly Isolated ATII Cells

Because CFTR expression decreases during cell culture, patch-clamp experiments were conducted on freshly isolated cells. Basal whole cell current in unstimulated cells typically stabilized within 2–3 min after formation of whole cell configuration, a time required for the pipette solution to diffuse into the cell. With CsCl solution in the bath and pipette, the basal currents observed from different cells were in the range between −30 and −150 pA at a test potential (V_{test}) of −100 mV. Mean cell membrane capacitance was 3.8 ± 1.9 pF (mean ± SD, n = 49). In some cells, however, despite the use of near isosmotic solutions, formation of whole cell configuration led to transient stimulation of cell swelling-activated Cl⁻ current. The current presented characteristic outward rectification and voltage-dependent inactivation at high positive voltage steps (data not shown). Such cells required up to 20 min for the current to return to baseline. The cells were stimulated with a cAMP-elevating cocktail only after stable basal currents were achieved. Increment of cellular cAMP resulted in a significant increase of whole cell conductance that peaked within 3–5 min, followed by a slow decay (Fig. 6, A and B). Approximately 44% of cells tested (16 out of 36) responded to cAMP. With CsCl solution in the bath and pipette, mean cAMP-stimulated current at the peak of the response was 101 ± 34 pA/cell (or 26.6 ± 8.9 pA/pF; n = 12, V_{rest} = −100 mV). This current was inhibited ~50% by the Cl⁻ channel blocker NPPB (100 μM, n = 6), whereas DIDS (250 μM, n = 4) was without effect, as might be expected for CFTR-mediated current (Fig. 6B, inset). This cAMP-stimulated whole cell conductance had a linear current-voltage (I-V) relationship and showed no time dependence during the 900-ms voltage steps. Its reversal potential (E_r) was shifted by approximately −20.8 ± 3.8 mV (n = 3) when Cl⁻ concentration in the pipette solution was reduced from 104 to 34 mM (Fig. 6C). This E_r shift was close to that expected from the Nernst equation (ΔE_r = −28 mV), and the difference may have arisen from several factors, including a small contribution of cation nonelective currents to the observed whole cell conductance. Thus our data are consistent with Cl⁻-selective conductance, with properties resembling those of CFTR Cl⁻ channels.

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cAMP Activates Low-Conductance Single Channel Currents in ATII Cells

To identify Cl⁻ channels underlying cAMP-activated whole cell conductance seen in freshly isolated ATII cells, we p-

![Fig. 5](image_url)

**Fig. 5.** Detection of CFTR protein in epithelial alveolar cells. Western blot analysis of whole cell lysate from NuLi 1 (lane 1, positive control, 50 μg of protein), BHK transfected with empty pNUT vector lacking CFTR cDNA (lane 2, negative control, 20 μg of protein), BHK transfected with human wt-CFTR (lane 3, positive control, 20 μg of protein), and 4 representative epithelial alveolar cell lysates (lanes 4–7, ATII #1–ATII #4, 120 μg of protein) obtained from 4 different rats.

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formed single channel, cell-attached recordings on cells stimulated with forskolin. To minimize currents through Na$^+$ and K$^+$ channels, we used CsCl solutions in the bath and pipette. Furthermore, we included 100 μM amiloride in the pipette solution to block currents through Na$^+$ and cation nonselective channels often noted in these cells (14, 21). Under such conditions, forskolin induced single-channel activity that had a slope conductance of ∼4 pS (Fig. 7). Single channel activity was recorded in 7 out of 16 attempts with some patches containing >1 active CFTR channel. Average channel open probability varied significantly (0.3–0.8) between different patches and with time after cAMP stimulation but was not voltage dependent.

DISCUSSION

In this study, we demonstrated the presence of CFTR in freshly isolated adult alveolar cells at the mRNA and protein levels as well as functional CFTR channels by the patch-clamp technique. The cell mix, obtained by the differential adherence method (6), contained ∼85% of ATII cells. The remaining cells likely included mainly macrophages and potentially some ATI cells. Macrophages, however, should not constitute a contamination for RT-PCR CFTR amplification since they do not express CFTR mRNA (20). This agrees with the absence of functional CFTR channels in macrophages, as revealed by our patch-clamp experiments. With RT-PCR, the highest level of CFTR expression was detected in freshly isolated ATII cells but dropped by ∼60% after 1 day of cell culture on permeable filters. The detection of CFTR mRNA in freshly isolated cells confirms earlier reports, which suggested the presence of this channel in the adult alveolar epithelium (11, 23, 44).

ATII cells are known to undergo phenotypic modification when they are kept in culture. Significant morphological changes, such as flattening of cells, loss of lamellar bodies, and decreased expression of the surfactant proteins SP-A, -B, and decreased expression of the surfactant proteins SP-A, -B, and rTI 40 (9) and aquaporin-5 (3). For this reason, in addition to the CFTR, we tested SP-A and rTI 40 mRNA expression in these cells. We found that SP-A expression, as well as alkaline phosphatase staining, decreased significantly in cultured cells (P < 0.05), whereas rTI 40 expression showed a concomitant increase (P < 0.05). Similar changes in CFTR and SP-A expression during culture of alveolar epithelial cells suggest that the CFTR channel is expressed primarily in ATII
cells. This would also explain why the strongest signal is detected in freshly isolated cells. These results confirm earlier reports that demonstrated by in situ hybridization the presence of the CFTR in cuboidal cells at the corner of alveoli where ATII cells are located (11, 44). Despite a reduced level, SP-A mRNA expression remained detectable after 4 days of culture on permeable filters. This is somewhat different from the result reported previously by Shannon et al. (46), where SP-A mRNA expression was lost in alveolar epithelial cells cultured on plastic. The difference can be explained by different culture conditions, i.e., filter vs. plastic, as well as by the higher sensitivity of the RT-PCR technique that we engaged instead of Northern blotting. In parallel to SP-A expression, it was noted that alkaline phosphatase activity, which gives intense staining in freshly isolated cells, decreased in primary cultured cells. This outcome is consistent with the observations of Edelson et al. (10) who reported that alkaline phosphatase expression, a marker of ATII cell differentiation, declines with time of cell culture.

Culturing cells at the ALI has been shown to greatly increase maintenance of the ATII cell phenotype (8) and to augment active ENaC at the cell surface (21). Because cells grown at the ALI also markedly heighten CFTR maturation and trafficking in MDCK cell monolayers (1), we tested whether such culture conditions would also enhance CFTR mRNA expression in alveolar epithelial cells. Cells grown under ALI conditions expressed significantly more CFTR mRNA compared with cells grown on submerged filters, although it remained 30% lower than in freshly isolated cells. In contrast to the CFTR, rTL40 and SP-A expression was not affected in the three culture conditions tested. As rTL40 and SP-A are specific markers of ATI and ATII cells, respectively, it suggests that the increase in CFTR expression detected under ALI conditions is not linked to cell differentiation per se.

In this study, we also investigated, by RT-PCR, CFTR transcript expression in trachea and distal lung tissues. More cycles were needed to amplify a CFTR signal from the lung than from purified alveolar epithelial cells. The signal was significantly stronger in the distal lung than in the trachea. This is not in contradiction with what has been reported by others, since, in rodents, very little CFTR mRNA has been found in the trachea (44). In rats, the CFTR has been detected in low levels at the epithelial surface of the bronchi and bronchioles (51). In situ hybridization in the human lung disclosed that CFTR mRNA was present from the bronchi to the alveoli (11). The CFTR has also been noted, although at a low level, in the corner of mouse alveoli (44). Detection of the CFTR in freshly isolated ATII cells and the large number of these cells in the lung suggest that a part of the CFTR signal in the distal lung could arise from ATII cells.
Our Western blot experiments showed the presence of a 164-kDa band, which was consistent with the molecular mass of the mature rat CFTR, as reported by Mulberg et al. (37) (150–165 kDa) and by Gong et al. (16) (145 kDa). A lower band (144 kDa) that could correspond to the unglycosylated form of the CFTR was also detected. This result confirmed the presence of CFTR at the protein level in rat epithelial alveolar cells. However, it is important to mention that a relatively large amount of protein (120 μg) was employed to detect the CFTR.

In addition, the observed level of CFTR expression was variable from animal to animal (Fig. 5). This observation could explain the variability in cell response to cAMP measured in whole cell experiments.

Our patch-clamp data demonstrate that under conditions where Na+ and K+ currents are abolished: 1) elevation of cellular cAMP stimulates whole cell Cl–-selective conductance; 2) this conductance has a linear I-V relationship; 3) it is suppressed by 100 μM NPPB, a Cl– channel blocker often used to inhibit the CFTR, but it is insensitive to 250 μM DIDS; and 4) in cell-attached experiments, with 100 μM amiloride in the pipette solution to block ENaC and other Na+–conducting channels, cAMP activated single channel currents of linear I-V relationship corresponding to ~4-pS slope conductance. By comparison, the human CFTR has a higher conductance, typically 7–9 pS in excised membrane patches with high Cl– concentration on both sides of the membrane (25). Lower conductance of the rat CFTR found here may be due to species differences and experimental conditions. For example, in cell-attached configuration, consistently lower single channel conductance of 3.6 pS was observed in cAMP-stimulated rat pancreatic ducts, thus closely matching the conductance recorded in our study (17). The CFTR of another rodent, mice, also has conductance significantly lower than that of humans (5.4–5.7 pS) (25). Furthermore, lower single channel conductance is typical in cell-attached configuration as a result of low intracellular Cl– concentration and/or inhibition by large intracellular anions (19, 49). Collectively, our patch-clamp data are consistent with the known biophysical and pharmacological properties of CFTR Cl– channels, demonstrating their presence in the plasma membrane of freshly isolated rat ATII cells.

In whole cell patch-clamp experiments, ~40% of cells responded to cAMP stimulation. This relatively low response rate might not be surprising, since our freshly isolated cell mix contained ~85% of ATII cells, as determined by alkaline phosphatase staining assay. Cell viability was certainly another limiting factor. Trypan blue exclusion assay indicated ~90% viability immediately after cell isolation, which declined to ~80% after cell suspension storage on ice for 3 h, the average time before cells were tested in our patch-clamp experiments. Furthermore, after the trauma of cell isolation, not all freshly isolated cells, which appear viable by trypan blue exclusion, will retain full responsiveness of their intracellular signaling pathways. Finally, the presence of ATII cell subpopulations with different CFTR expression levels may also contribute to the observed variability of cell responses to cAMP stimulation.

It is noteworthy that in whole cell experiments with macrophages, a major contaminant of our cell mix, no responses to cAMP were seen; therefore, the majority of responses that we observed could be attributed to alveolar cells.

A previous patch-clamp study by Zhu et al. (53) did not find evidence of CFTR channels in adult rat ATII cells. However, they did not test freshly isolated cells, but rather cells cultured on a glass substrate. This may have had an impact on CFTR expression, as discussed above. In another study, CFTR-like, cAMP-activated, outwardly rectifying whole cell Cl– currents were detected in freshly isolated and cultured ATII cells (23). The presence of the CFTR in the apical membrane of cultured ATII cells in that study was further supported by immunofluorescence labeling of the CFTR. However, interpretation of their patch-clamp data is complicated by the outward rectification of whole cell currents, which was observed with both low and high intracellular Cl– solutions (23). Such rectification is not expected for the CFTR, at least in symmetrical, high Cl– solutions, but is characteristic of volume-sensitive Cl– channels (48), which may have contributed to the whole cell currents recorded.

The presence of functional CFTR in adult alveolar cells revealed by our study should help us to better understand its role in lung liquid balance and in the pathophysiology of cystic fibrosis. Indeed, recent investigations have implicated CFTR-mediated Cl– transport in the modulation of cAMP/terbutaline-stimulated Na+ absorption (26, 41) and in lung liquid clearance (12). In the latter study, isoproterenol increased in vivo fluid clearance in distal air spaces in wild-type mice, but the response was absent in cystic fibrosis ΔF508 mice (12). Our experiments demonstrating functional CFTR in adult ATII cells should strengthen these observations. Furthermore, the CFTR may also contribute to the secretion of pulmonary liquid in the adult lung. Indeed, the maintenance of a thin liquid layer at the surface of lung epithelial cells plays a critical role in the pathogenesis of cystic fibrosis (4). The site of this secretion remains unknown, but it has been proposed to take place in the distal region of the lungs and move up the airway surface (4, 42, 44). If liquid forms in the alveoli because of the large surface of this epithelium, low-level Cl– and fluid transport rates would be sufficient for secretion of the required lung liquid volume. Assuming an open state probability of 1 for activated CFTR channels, one could estimate from the observed cAMP-stimulated whole cell conductance of 300–1,500 pS/cell that there are at least 50–250 CFTR channels per ATII cell. Such a relatively low number of CFTR copies is expected in physiological settings in respiratory epithelial cells (42).

In summary, our study provides direct evidence for the presence of functional CFTR Cl– channels in adult ATII cells, where they could contribute to lung liquid homeostasis. Further work is required to elucidate the precise role of CFTR and other ion transport pathways in this process.

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