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Interdependency of β -Adrenergic Receptors and CFTR in Regulation of Alveolar Active Na^+ Transport

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Abstract— β -Adrenergic receptors (β AR) regulate active Na^+ transport in the alveolar epithelium and accelerate clearance of excess airspace fluid. Accumulating data indicates that the cystic fibrosis transmembrane conductance regulator (CFTR) is important for upregulation of the active ion transport that is needed to maintain alveolar fluid homeostasis during pulmonary edema. We hypothesized that β AR regulation of alveolar active transport may be mediated via a CFTR dependent pathway. To test this hypothesis we used a recombinant adenovirus that expresses a human CFTR cDNA (adCFTR) to increase CFTR function in the alveolar epithelium of normal rats and mice. Alveolar fluid clearance (AFC), an index of alveolar active Na^+ transport, was 92% greater in CFTR overexpressing lungs than controls. Addition of the Cl^- channel blockers NPPB, glibenclamide, or bumetanide and experiments using Cl^- free alveolar instillate solutions indicate that the accelerated AFC in this model is due to increased Cl^- channel function. Conversely, CFTR overexpression in mice with no β_1 - or β_2 -adrenergic receptors had no effect on AFC. Overexpression of a human β_2 AR in the alveolar epithelium significantly increased AFC in normal mice but had no effect in mice with a non-functional human CFTR gene ($\Delta\phi 508$ mutation). These studies indicate that upregulation of alveolar CFTR function speeds clearance of excess fluid from the airspace and that CFTRs effect on active Na^+ transport requires the β AR. These studies reveal a previously undetected interdependency between CFTR and β AR that is essential for upregulation of active Na^+ transport and fluid clearance in the alveolus. (*Circ Res.* 2005;96:999-1005.)

Key Words: pulmonary edema ■ cystic fibrosis transmembrane conductance regulator ■ alveolar fluid clearance ■ chloride channel ■ β -adrenergic receptors

β -Adrenergic receptors (β AR) on alveolar epithelial cells upregulate active Na^+ transport and accelerate clearance of excess fluid from the alveolar airspace. Data from in vitro and in vivo models indicate that β AR signaling increases epithelial Na^+ channel number and open probability and promotes transcription and trafficking of Na,K-ATPases to the cell membrane of alveolar epithelial cells.¹⁻³ Maintenance of electroneutrality across the alveolar epithelium requires that Cl^- exit the airspace via trans- and paracellular pathways.⁴ Accumulating data from in vitro and in vivo studies indicate that the cystic fibrosis transmembrane conductance regulator (CFTR) is present in alveolar epithelial cells and provides a path for Cl^- transport to clear excess alveolar fluid, although it may not be needed for maintenance of alveolar fluid balance in the normal/uninjured lung.⁵

We have recently reported that the β_2 AR is essential for upregulation of alveolar active Na^+ transport.⁶ In these studies, we observed that β_2 AR signaling is required to

maintain normal expression or function of amiloride-sensitive Na^+ transport and basolateral Na,K-ATPase function in the distal mouse lungs. Data from both airway and alveolar epithelial cells indicate that β AR signaling increases Cl^- flux through CFTR, which is present at low levels in the apical cell membrane of human and rodent alveolar epithelial cells.^{7,8} Single cell and monolayer studies of rat and rabbit alveolar epithelial cells indicate that these cells can reabsorb Cl^- through a cAMP-sensitive pathway.^{8,9} This body of data led us to hypothesize that CFTR may play a role in β_2 AR-mediated effects on alveolar active Na^+ transport.

In the present study, we tested the effects of upregulation of CFTR function on alveolar active Na^+ transport in normal rats and mice, and mice with targeted deletions of the β AR or with a nonfunctional human CFTR gene (CFTR $\Delta\phi 508$). These studies reveal an interdependency between CFTR and the β_2 AR where both proteins are required to affect increases in β AR-driven alveolar active Na^+ transport.

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Materials and Methods

Adenovirus Propagation and Purification

Replication-incompetent E1a⁻/E3⁻ human type 5 adenoviruses containing either a full-length human CFTR cDNA under the control of a human CMV immediate-early promoter enhancer gene (adCFTR, from Drs M. Welsh and J. Zabner, University of Iowa, Iowa City, Iowa), a human β_2 AR cDNA under the control of a human CMV immediate-early promoter enhancer gene (ad β_2 AR, from Drs R. Lefkowitz and W. Koch, Duke University, Durham, NC), or no cDNA (adNull) were propagated and purified as previously described.^{10,11} All viruses used in this study were from single preparations and were free of signs of replication-competent adenovirus to a maximum dilution of 10^9 .

Animals

The use of animals for this study was approved by the Evanston Northwestern Healthcare (ENH), Northwestern University, and Columbia University Institutional Animal Use and Care Committees. Mice homozygous for deletions of both the β_1 - and β_2 -adrenergic receptors (β_1 AR^{-/-}/ β_2 AR^{-/-}) and strain-specific β_1 AR^{+/+}/ β_2 AR^{+/+} control mice (C57Bl6/J-DBA/2-129SvJ) were provided by Dr B. Kobulka (Stanford University, Stanford, Calif).¹² Transgenic mice homozygous for the $\Delta\phi 508$ mutation in the CFTR gene (CFTR^{-/-}) and wild-type (CFTR^{+/+}) littermate controls were from Dr J. Engelhardt (University of Iowa, Iowa City, Iowa).¹³ Concurrently maintained sentinel animals were without signs of specific viral or bacterial pathogens. C57bl6 mice were from Harlan Laboratories, Indianapolis, Ind. All mice used in these studies were 8- to 12-week-old males weighing 20 to 25 g. Rats used in this study were adult, male Sprague-Dawley rats (Harlan Laboratories) weighing 275 to 300 g.

Adenovirus Delivery to Rat and Mouse Lungs

Adenovirus was delivered to rats and mice using previously described methods.^{6,10,14} Briefly, these methods use a surfactant-based vehicle that produces transgene expression that is confined to the alveolar epithelium. Rats received 5×10^{10} viral particles and mice 1×10^{10} viral particles. In all experiments, mice and rats infected with ad β_2 AR or adCFTR were compared with rats treated with vehicle alone (sham) or infected with a first generation adenovirus that expresses no transgene (adNull). This strategy and inclusion of 7 days of after infection recovery has proven effective in controlling for the effects of the host response to these adenovectors in prior studies.^{6,10,15-17} We have previously shown that sham infection has no measurable effect on alveolar fluid clearance (AFC) in rats and mice; thus, uninfected controls were not used in this study.^{6,10,15-18}

Measurement of AFC in Isolated Rat Lungs

The isolated lung preparation used in this study has been previously used to measure alveolar active Na⁺ transport in rats infected with adenoviruses.^{10,15-17,19-21} Briefly, change in concentration of a protein marker (Evan's blue tagged albumin) instilled into the airspace compartment of isolated and perfused rat lungs is used to calculate change in alveolar volume over a 60-minute experimental period. Fluorescence isothiocyanate (FITC)-tagged albumin was added to the perfusate to monitor leakage of protein from the vascular compartment into the airspace to produce an index of alveolar permeability.

Rat Treatment Protocols

AFC was measured in sham, adNull, and adCFTR-infected rats (9 animals/group). An additional 5 rats per group were studied with amiloride (10^{-6} mol/L) in the alveolar instillate during clearance measurements. To estimate Cl⁻ channel function in sham, adNull, and adCFTR-infected rat lungs the Cl⁻ transport inhibitors 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB, 10^{-4} mol/L), glibenclamide (10^{-4} mol/L), or bumetanide (10^{-4} mol/L) were individually added to the alveolar instillate during clearance measurements. The Na⁺ channel blocker amiloride (10^{-6} mol/L) was included in the alveolar instillate in some experiments.

AFC Measurements in Live Mice

The method of measurement used in this study was modified from Hardiman et al and has been described elsewhere.^{6,22,23} This method is similar to that used in rats except that 300 μ L of Evan's blue-tagged albumin is instilled into lungs of sedated, mechanically ventilated mice that are maintained supine, and clearance is measured over a 30-minute period. Assessment of Cl⁻ channel function in mice was via use of a Cl⁻-free instillate solution where NaCl was replaced with equimolar amounts of NaGluconate without altering osmolarity. In some mice, forskolin (10^{-4} mol/L) was included in the instillate solution during clearance measurements. Experiments using Cl⁻-free instillate were performed with a positive end-expiratory pressure of 3 cmH₂O above ambient pressure using a water valve attached to the expiratory port of a rodent ventilator.

Reverse Transcriptase PCR

Total RNA from peripheral (distal 2 to 3 mm) and proximal (central airways) lung tissue and liver was isolated using RNA-zol B (Tel-Test, Inc). RNA was treated with DNase I (50 U, Sigma), twice extracted with phenol-chloroform, precipitated, and resuspended in 20 μ L of RNase-free water before first strand cDNA synthesis using oligo-dT₁₂₋₁₆. PCR was performed using 2 μ L of the reaction mixture, 30 cycles of amplification, and an annealing temperature of 58°C. Primer sequences used to detect human CFTR mRNA were as follows: upstream 5'ACGCTTCAGGCACGAA and downstream 5'CGGCTACTCCCACGTAA.

Cell Membrane Isolation

Whole-cell membrane proteins and membrane fractions enriched for the basolateral membrane domain were produced from tissue collected from the distal 2 to 3 mm of right rat lungs after serial bronchoalveolar lavage and perfusion of the pulmonary artery with PBS, as previously described.^{10,16,17,24}

Western Analysis

Twenty micrograms of whole-cell membrane protein was separated with 4% to 12% SDS-PAGE (Invitrogen Life Technology), electrophoretically transferred to nitrocellulose and probed with a mouse monoclonal anti-human CFTR antibody (R&D Systems), a rabbit anti-rat ENaC antibody (Affinity Bioreagents), a rabbit anti-Na,K-ATPase α_1 antibody (K. Geering, University of Lausanne, Switzerland)²⁵ or a mouse monoclonal anti-actin antibody (Chemicon International). Protein bands were visualized using peroxidase coupled secondary antibodies and a chemiluminescence detection kit (Pierce).

Na,K-ATPase Function (P_i Liberation From ATP) in the Distal Lung

Twenty micrograms of basolateral cell membrane protein isolated from the peripheral lung (distal 2 to 3 mm) of rats was resuspended in 100 μ L of a high [Na⁺]/low [K⁺]/high [ATP] reaction buffer (in mmol/L: 50 Tris-HCl pH 7.4, 50 NaCl, 5 KCl, 10 Mg₂Cl, 1 EGTA, 10 Na₂ATP), with [γ -³²P]-ATP (3.3 μ Ci/mL) as previously described.^{16,17} These conditions maximize Na,K-ATPase activity (V_{max}) and produce an index of functional, membrane-bound receptor number. Triplicate samples were placed at -20°C for 15 minutes before incubation for 15 minutes at 37°C. The reaction was terminated by the addition of 5% TCA/10% charcoal and cooling to 4°C. The charcoal phase containing unhydrolyzed nucleotide was separated by centrifugation (12 000g for 5 minutes) and the liberated ³²P quantified. Na,K-ATPase activity was calculated as the difference between the test samples (total/nonspecific ATPase activity) and samples assayed in reaction buffer with 2.5 mmol/L ouabain but devoid of Na⁺ and K⁺ (non-Na,K-ATPase ATPase activity). Results are expressed as mean nmol of P_i/mg of protein per hour of triplicate measurements from 3 rats per group.

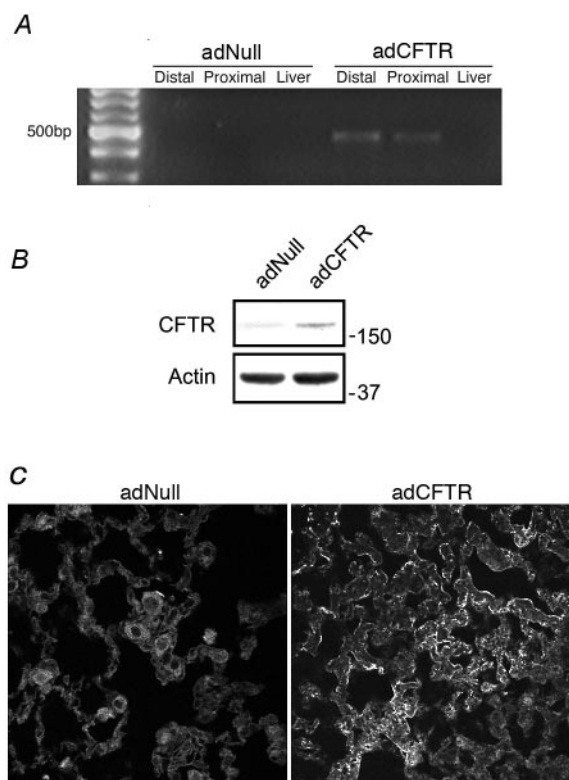


Figure 1. Human CFTR mRNA expression in rat lung. A, Reverse transcriptase PCR of distal lung tissue (outer 2 to 3 mm), central lung tissue (trachea and mainstem bronchi) and liver from rats infected with adNull or adCFTR 7 days before study. B, Western analysis of peripheral lung tissue from rats infected with adNull or adCFTR showing increased expression of CFTR protein. C, Confocal images of adNull- and adCFTR-infected C57b6 mouse lungs immunostained using anti-human CFTR primary antibodies (original magnification, 60 \times).

Immunohistochemistry for CFTR

Mouse lungs (C57b6) were fixed in 2% formaldehyde/0.2% glutaraldehyde imbedded in paraffin and sectioned (5 μ m). Deparaffinized and rehydrated sections were blocked at room temperature for 1 hour with 5% normal goat serum in Superblock before incubation with anti-CFTR antibodies (13-1 and 24-1, R&D Systems, 1:100 dilution; MM13-4 and M3A7, Upstate USA, Inc. 1:200 dilution) at 37 $^{\circ}$ C for 2 hours and then washed 5 times with PBS. Immunodetection was with Alexa568 (Molecular Probe, 1:1000) for 1 hour (at 37 $^{\circ}$ C) followed by washing 5 times with PBS. The slides were then mounted with Vectashield (Vector Labs, Inc).

Data Analysis

All values are reported as mean \pm standard deviation (SD). Statistical significance was defined as $P < 0.05$.

Results

Transgene Expression

To produce a model of alveolar CFTR overexpression, a first generation adenovirus that expresses a human CFTR cDNA under the control of a human CMV promoter-enhancer element (adCFTR) was used to transduce the lungs of normal rats (Figure 1). This vector has been shown to increase CFTR function in airway epithelial cells, animal models, and humans with cystic fibrosis.^{26–29} Human CFTR mRNA was detected in both proximal and distal rat lung tissue 7 days after infection with adCFTR using rtPCR and human CFTR-

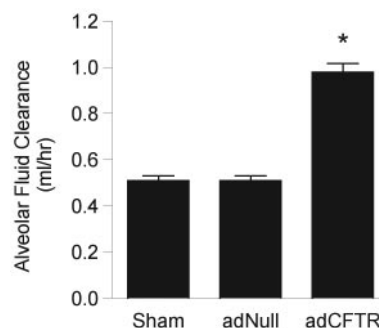


Figure 2. Effect of CFTR overexpression on AFC in normal rats. Rate (mL/hour) of fluid removed from the alveolus of sham-, adNull-, and adCFTR-infected rats. $n = 9$ rats/group. * $P < 0.002$ adCFTR vs sham and adNull.

specific primers (Figure 1A). No human CFTR mRNA was observed in livers of adCFTR-infected rats or in lungs and livers from adNull-infected controls. Increased CFTR protein expression was noted in cell membranes isolated from the distal lungs of adCFTR-infected rats (Figure 1B). Immunostaining of adCFTR-infected C57b6 mouse lungs using anti-human CFTR primary antibodies demonstrated patchy immunoreactivity in a linear pattern that surrounded the airspace, consistent with transduction of all alveolar epithelial cells (Figure 1C).

Effects of CFTR Overexpression on AFC in Rats

AFC in rats infected with adCFTR (5×10^1 viral particles) was 92% greater (0.98 ± 0.11 mL/hour) than adNull (0.51 ± 0.06 mL/hour) and sham-infected controls (0.51 ± 0.02 , $P < 0.002$ adCFTR versus sham and adNull) (Figure 2). No flux of albumin from the pulmonary vasculature into the airspace was measurable in any of the experimental groups implying that alveolar barrier function was not altered by gene transfer or CFTR overexpression. To test if CFTR gene transfer affects alveolar Cl^- channel function, we conducted experiments using 3 established Cl^- channel inhibitors in the alveolar instillate during clearance measurements. NPPB did not affect AFC in sham- and adNull-infected lungs, whereas glibenclamide and bumetanide reduced it by 20% (Figure 3A through 3C). Glibenclamide, bumetanide, and NPPB reduced AFC in adCFTR-infected lungs by 55% to 60%. Net clearance in adCFTR-infected lungs treated with these agents was the same as similarly treated sham- and adNull-infected controls; thus, Cl^- channel blockade completely negated the effects of CFTR overexpression.

To further evaluate if CFTR gene transfer alters epithelial Cl^- channel function, AFC was measured using an instillate solution free of Cl^- (Figure 3D). Doing so resulted in Cl^- flux along its concentration gradient from the interstitial into the alveolar airspace and has been shown to cause fluid accumulation in the airspace.⁴ These experiments were conducted using C57bl6 mice and a positive end-expiratory pressure of 3 cmH_2O , which increased basal AFC to $30.29 \pm 4.5\%/30$ minutes. This was substantially greater than that reported previously by our group⁶ but is consistent with recent data from Hardiman.²³ The absence of Cl^- caused airspace fluid accumulation (AFC: $-8.8 \pm 3.7\%/30$ minutes) that doubled

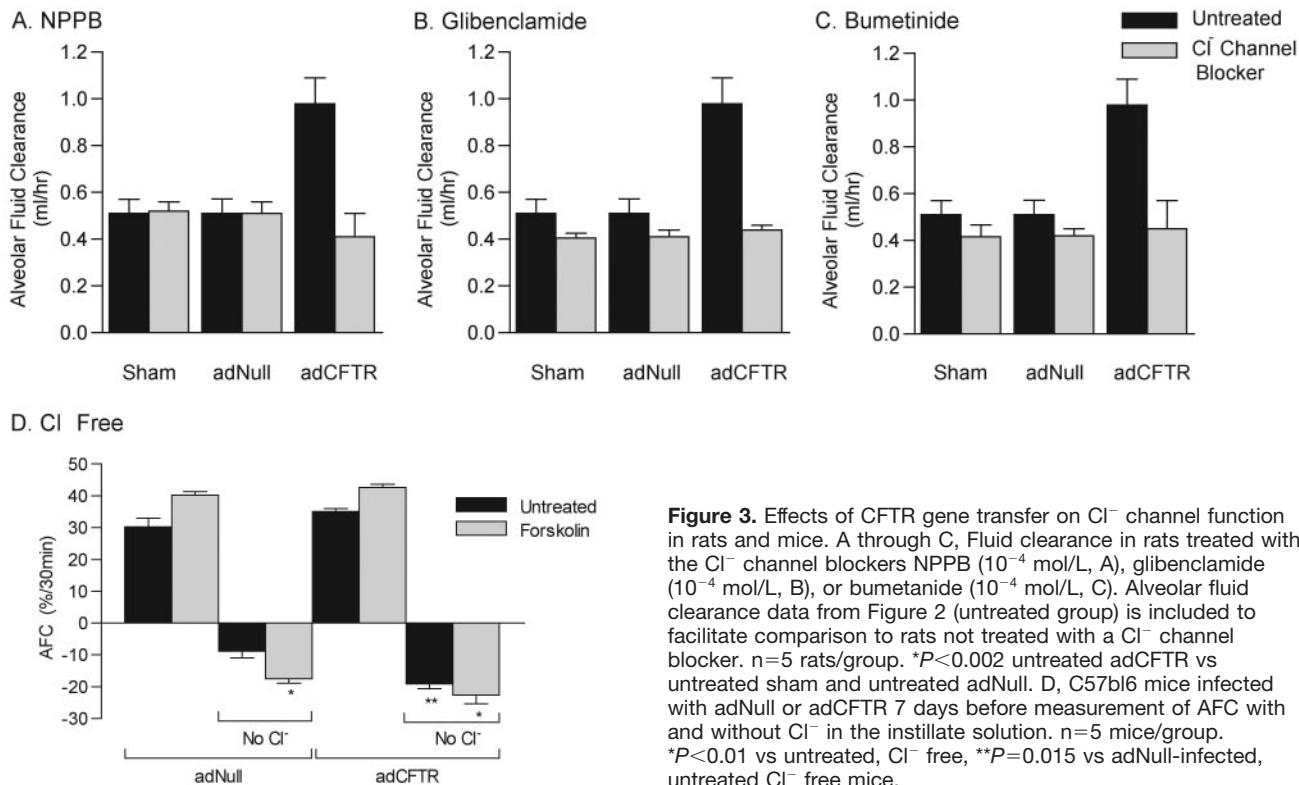


Figure 3. Effects of CFTR gene transfer on Cl⁻ channel function in rats and mice. A through C, Fluid clearance in rats treated with the Cl⁻ channel blockers NPPB (10⁻⁴ mol/L, A), glibenclamide (10⁻⁴ mol/L, B), or bumetanide (10⁻⁴ mol/L, C). Alveolar fluid clearance data from Figure 2 (untreated group) is included to facilitate comparison to rats not treated with a Cl⁻ channel blocker. n=5 rats/group. *P<0.002 untreated adCFTR vs untreated sham and untreated adNull. D, C57bl6 mice infected with adNull or adCFTR 7 days before measurement of AFC with and without Cl⁻ in the instillate solution. n=5 mice/group. *P<0.01 vs untreated, Cl⁻ free, **P=0.015 vs adNull-infected, untreated Cl⁻ free mice.

to $-17.5 \pm 2.8\%/30$ minutes when the adenylyl cyclase activator forskolin was included in the instillate of adNull-infected mice. Basal and forskolin-stimulated AFC in adCFTR mice was significantly more negative than adNull ($-19.1 \pm 2.7\%/30$ minutes and $-22.7 \pm 5.2\%/30$ minutes, respectively). These findings are consistent with increased function of a cAMP sensitive Cl⁻ channel.

Effects of CFTR Overexpression on Na⁺ Channel and Na,K-ATPase in Rats

Amiloride (10⁻⁶ mol/L) was included in the alveolar instillate during clearance measurements to assess amiloride-sensitive Na⁺ channel function. As can be seen in Figure 4A, amiloride reduced AFC to a greater degree in adCFTR-infected lungs than in sham- and adNull-infected controls (Δ AFC: adCFTR,

-57% to 0.42 ± 0.11 mL/hour; adNull, -46% to 0.26 ± 0.03 mL/hour; sham, -49% to 0.27 ± 0.07 mL/hr) ($P < 0.05$ adCFTR versus adNull or sham) (Figure 4A). However, the absolute clearance rate in amiloride-treated adCFTR-infected lungs (0.42 ± 0.11 mL/hour) remained greater than similarly treated sham (0.27 ± 0.07 mL/hour) and adNull controls (0.26 ± 0.03 mL/hour) ($P < 0.03$ adCFTR versus sham or adNull). Western analysis for Epithelial Na⁺ Channel α subunit (α ENaC) using whole-cell membranes from the peripheral lung did not reveal significant differences in expression between adNull and adCFTR infected lungs (Figure 4B). Thus, CFTR gene transfer increases both amiloride-sensitive and insensitive Na⁺ channel function in rats. Whether this is due to changes in ENaC expression, the biophysical properties/amiloride sensitivity of ENaC, or up-regulation of an amiloride-insensitive apical Na⁺ entry pathway is not yet known.

The effect of CFTR gene transfer on Na,K-ATPase activity was assessed by measuring ouabain-sensitive liberation of inorganic phosphate (P_i) from ATP in the presence of substrate independent conditions (high [Na⁺]/low [K⁺]/high [ATP]) that allow the Na⁺ pump to function maximally (V_{max}). Under these conditions, the determinate of P_i release is the number of functional Na,K-ATPases in basolateral membranes from the peripheral lung. AdCFTR-infected rat lungs had up to 66% more functional Na,K-ATPases in basolateral membranes than sham- and adNull-infected controls (2.91 ± 0.32 , 1.57 ± 0.23 , and 1.75 ± 0.42 nmol P_i/mg protein per hour, respectively; $P < 0.02$ adCFTR versus sham and adNull) (Figure 5A). Western analysis likewise showed increased expression of the Na,K-ATPase α_1 subunit in

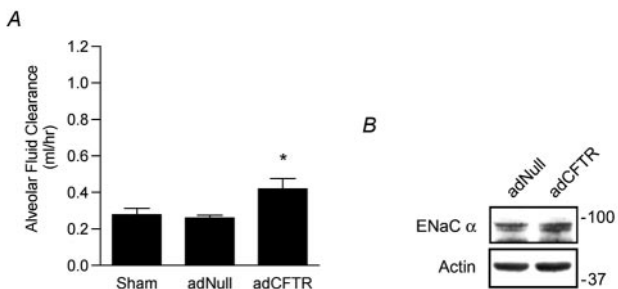


Figure 4. Na⁺ channel expression and function. A, AFC was measured in the presence of the Na⁺ channel blocker amiloride (10⁻⁶ mol/L) in the instillate. n=5 rats/group. *P<0.03 adCFTR+amiloride vs sham or adNull infected+amiloride. B, Immunoblot of whole-cell membranes (20 μ g protein/lane) from peripheral lung of adNull and adCFTR infected rats probed with an anti- α ENaC antibody.

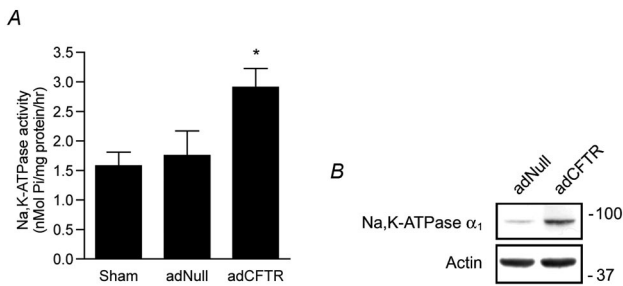


Figure 5. Peripheral lung Na,K-ATPase function. A, Ouabain-sensitive liberation of inorganic phosphate from ATP (Na,K-ATPase activity) by basolateral membranes isolated from the peripheral lung of sham-, adNull-, and adCFTR-infected rats. $n=3$ rats/group. $*P<0.02$ adCFTR vs sham and adNull. B, Immunoblot of whole cell membranes (20 μ g protein/lane) from peripheral lung of adNull- and adCFTR-infected rats probed with an anti- α_1 Na,K-ATPase antibody.

adCFTR-infected lungs (Figure 5B). These findings indicate that upregulation of Cl^- channel function results in parallel changes of other transport proteins.

CFTR Gene Transfer to Alveoli of Mice With Targeted Deletions of the β_1 - and β_2 -Adrenergic Receptors

We have recently reported that mice with targeted deletions of the β_1 and β_2 adrenergic receptors ($\beta_1AR^{-/-}/\beta_2AR^{-/-}$) have decreased basal AFC, Na,K-ATPase function, and amiloride sensitivity.⁶ Rescue of alveolar epithelial β_2AR function normalizes these processes confirming that these mice retain G-protein signaling capability and can upregulate lung ion transport proteins. To test if changes in active transport after CFTR gene transfer require β_2AR function, we infected mice with 1×10^{10} viral particles of adCFTR 7 days before study using a recently described surfactant-based delivery system.⁶ Fluid clearance in this model was measured using a “live” mechanically ventilated intact lung model that preserves oxygenation, ventilation, and serum pH.^{22,23} CFTR gene transfer increased AFC in strain-specific $\beta_1AR^{+/+}/\beta_2AR^{+/+}$ mice by 36% (from $22.2 \pm 3.0\%$ to $30.3 \pm 2.9\%$; $P<0.02$ versus uninfected, sham, and adNull infected $\beta_1AR^{+/+}/\beta_2AR^{+/+}$; Figure 6A). This increase was less than that noted in rats (Figure 2) and is likely due to lower transduction efficiency (70%⁶ versus $>90\%$ ^{10,14}) and perhaps decreased dependence on CFTR for AFC in mice than rats. Importantly, CFTR gene transfer to the alveolar epithelium of $\beta_1AR^{-/-}/\beta_2AR^{-/-}$ mice had no effect on AFC measured 7 days after gene transfer. AdNull infection did not affect AFC at this postinfection time-point in either the $\beta_1AR^{+/+}/\beta_2AR^{+/+}$ or

$\beta_1AR^{-/-}/\beta_2AR^{-/-}$ groups. Clearance in these groups was the same as untreated/uninfected mice in a prior study.²

β_2AR Gene Transfer to the Alveoli of Mice With a Nonfunctional Human CFTR

To further approach the question of a functional interaction between β_2AR and CFTR, we transduced the alveolar epithelium of mice that have no mouse CFTR gene ($CFTR^{-/-}$) and are homozygous for a nonfunctional human CFTR gene ($\Delta\phi 508$ mutation) with an adenovirus that expresses a human β_2AR gene (ad β_2AR) (Figure 6B). We have shown that this approach increases β_2AR expression only in the alveolar epithelium and upregulates β_2AR function and alveolar active Na^+ transport in both wild-type and $\beta_1AR^{-/-}/\beta_2AR^{-/-}$ mice.⁶ Increased alveolar β -receptor function in this model appears to be due to increased numbers of β_2AR s in the cell membrane and enhanced sensitivity to endogenous catecholamines. As can be seen in Figure 6B, β_2AR gene transfer did not affect AFC in $CFTR^{-/-}$ mice but increased it by 42% in wild-type controls (from $21.7 \pm 4.2\%$ to $30.7 \pm 5.6\%$; $P=0.04$ versus sham-infected $CFTR^{+/+}$). Clearance in sham- and adNull-infected $CFTR^{-/-}$ mice was similar to sham (Figure 6B) and uninfected² wild-type mice consistent with other reports showing that CFTR may not be required for maintenance of basal alveolar active Na^+ transport.³⁰ These data, combined with that from experiments of CFTR gene transfer to $\beta_1AR^{-/-}/\beta_2AR^{-/-}$ mice suggest that β_2AR mediates increases in alveolar active Na^+ transport via a CFTR-dependent pathway and that CFTR function requires the presence of the β_2AR .

Discussion

In the current study, we tested if upregulation of CFTR function, via adenoviral-mediated overexpression, alters alveolar active Na^+ transport in rodents. To affect this goal, we used previously tested adenovectors and established delivery strategies that produce transgene expression in both type 1 and 2 alveolar epithelial cells in rats and mice (Figure 1C).¹⁶ Data from other groups indicates that the β_2AR is present in both type 1 and 2 epithelial cells,^{30,31} thus it is likely that the methods used in these experiments result in concomitant expression of CFTR and β_2AR in both types of alveolar epithelial cells.

The functional effects of CFTR gene transfer in rats were assessed by measuring the capacity of the lung to clear fluid instilled into the lung using a well-described isolated rat lung model.¹⁰ This experimental design is based on the principal that AFC is an index of active, vectorial Na^+ transport by

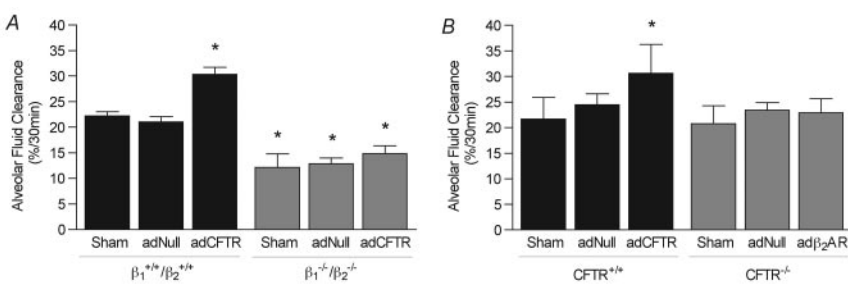


Figure 6. Effects of CFTR or β_2AR gene transfer in β -receptor knockout mice or $\Delta\phi 508$ ($CFTR^{-/-}$) mice. A, AFC was measured in live, mechanically ventilated $\beta_1AR^{+/+}/\beta_2AR^{+/+}$ and $\beta_1AR^{-/-}/\beta_2AR^{-/-}$ mice 7 days after sham, adNull, or adCFTR infection of mice. $n=4$ mice/group. $*P<0.02$ vs sham infected wild type. B, AFC in $CFTR^{+/+}$ and $CFTR^{-/-}$ mice 7 days after sham, adNull, or ad β_2AR infection. $n=4$ mice/group. $*P<0.04$ vs wild-type sham.

alveolar epithelial cells. Using this approach, we observed that overexpression of a human CFTR increases alveolar active Na⁺ transport by nearly 100% in normal rats (Figure 2) and by 42% in mice (Figure 6B). Inclusion of Cl⁻ channel blockers in the alveolar instillate during clearance measurements obviated the effects of CFTR overexpression (Figure 3). None of the blocking agents used is perfectly specific for CFTR, thus additional studies were conducted in mice using a Cl⁻-free instillate. This model results in Cl⁻ and fluid flux into the airspace and can be used to assess Cl⁻ channel function.⁴ Using this method, we noted that airspace fluid accumulation was greater in adCFTR-infected mice than in untreated and forskolin-treated adNull controls (Figure 3D). The inclusion of the adenylyl cyclase activator forskolin in the airspace of adCFTR-infected mice produced significant incremental fluid accumulation consistent with increased function of a cAMP-regulated Cl⁻ channel such as CFTR.

To probe how CFTR overexpression effects Na⁺ channel function, we included amiloride in the alveolar compartment during clearance measurements in rats (Figure 4A). Amiloride reduced alveolar active Na⁺ transport in lungs overexpressing CFTR, implying that increased CFTR function results in increased amiloride-sensitive Na⁺ channel (ie, epithelial Na⁺ channel) function (Figure 4A). Absolute clearance rates in amiloride-treated adCFTR lungs were greater than in similarly treated controls, suggesting that CFTR gene transfer also upregulates amiloride-insensitive Na⁺ entry pathways (Figure 4A). CFTR-infected rats also had significant increases in the number of functional Na,K-ATPases in basolateral cell membranes (Figure 4B). How then does CFTR overexpression increase vectorial ion transport? We speculate that overexpression of CFTR increases the number of open/activated CFTRs in the cell membrane—enhancing Cl⁻ entry and results in upregulation of amiloride-sensitive and insensitive Na⁺ entry pathways. This hypothesis requires that CFTR activate ENaC and other Na⁺ entry pathways and is in contradistinction to data from MDCK cells.³² Importantly, our data suggests that the interaction of CFTR with apical Na⁺ channels in alveolar epithelial cells might be markedly different than in proximal airway epithelial cells. Irrespective of this speculation, increased Na⁺ flux in the setting of CFTR overexpression results in upregulation of basolateral Na,K-ATPase capacity. The net result of these effects is increased vectorial Na⁺ flux and accelerated AFC.

Both β_1 - and β_2 -adrenergic agonists upregulate alveolar active Na⁺ transport in experimental models¹⁶ and humans.^{33,34} These agonists increase trafficking of Na,K-ATPases to the basolateral cell membrane, cause trafficking to the cell membrane and opening of epithelial Na⁺ channels, and increase Cl⁻ flux through CFTR.² To probe for a functional interaction between alveolar β AR and CFTR, we tested the effects of CFTR overexpression in mice with targeted deletions of both the β_1 - and β_2 - adrenergic receptor (β_1 AR^{-/-}/ β_2 AR^{-/-}). We have previously shown that these mice have normal total lung cAMP levels and retain G-protein signaling and adenylyl cyclase function but have much reduced basal alveolar active Na⁺ transport rates that can be normalized via rescue of β_2 AR function into the alveolar epithelium.⁶ CFTR overexpression in β_1 AR^{-/-}/

β_2 AR^{-/-} mice had no effect on alveolar active Na⁺ transport (Figure 6A). Thus, CFTR overexpression, in and of itself, does not alter vectorial ion flux across the alveolar epithelium. This observation caused us to test the effect of β_2 AR gene transfer to the alveolar epithelium of $\Delta\phi$ 508 mice. These crucial studies revealed that β_2 AR gene transfer in the absence of functional CFTR does not accelerate alveolar active Na⁺ transport as it does in wild-type controls. Recently, Fang and colleagues³⁵ reported that CFTR^{-/-} mice are unable to compensate for increased lung water in a model of hydrostatic pulmonary edema and, importantly, do not accelerate alveolar active Na⁺ transport in response to β -agonist treatment. We believe that the data from the present study and that of Fang support a new paradigm where CFTR and the β AR are functionally interdependent and both are required to upregulate alveolar active Na⁺ transport in the setting of pathological increases of fluid in the alveolar airspace. Presumptive mechanisms for this interaction have been suggested by Naren and colleagues who demonstrated that in airway epithelial cells (calu-3) β_2 ARs physically associate with CFTR through the PDZ domain of ezrin/radixin/moesin-binding phosphoprotein 50.³⁶ The formation of this macromolecular complex facilitates access of cAMP-activated protein kinase to R domain sites on CFTR and is required for cAMP-mediated regulation of CFTR.

The findings of this study raise the possibility that Cl⁻ transport may be a rate-limiting step for vectorial ion transport in the alveolar epithelium. While possible, we believe that alveolar active transport requires several key transport proteins (eg, ENaC, CFTR, Na,K-ATPase) that must interact to generate a transepithelial NaCl gradient, and that impairment of any of these protein families will impede AFC. Likewise, based on data from other studies, upregulation of single member of the transport pathway results in compensatory augmentation of other pathway components.^{6,10,11,16–18}

The present study provides new evidence that CFTR is an important contributor to the pathway by which the lung clears fluid from the alveolar airspace and that there exists an interdependence between β_2 AR and CFTR where both are required to upregulate alveolar active Na⁺ transport. These studies raise concern that the alveolar epithelium of patients with cystic fibrosis may lack the capacity to upregulate alveolar active Na⁺ transport in response to lung injury. The results of this study also suggest that upregulation of alveolar CFTR function might be an adjunct for improving the lung's ability to clear pulmonary edema.

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