Normalization of Raised Sodium Absorption and Raised Calcium-mediated Chloride Secretion by Adenovirus-mediated Expression of Cystic Fibrosis Transmembrane Conductance Regulator in Primary Human Cystic Fibrosis Airway Epithelial Cells

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Abstract

Cystic fibrosis airway epithelia exhibit a spectrum of ion transport properties that differ from normal, including not only defective cAMP-mediated Cl^- secretion, but also increased Na^+ absorption and increased Ca^{2+}-mediated Cl^- secretion. In the present study, we examined whether adenovirus-mediated (Ad5) transduction of CFTR can correct all of these CF ion transport abnormalities. Polarized primary cultures of human CF and normal nasal epithelial cells were infected with Ad5-CB*CFTR* at an moi (10^4) which transduced virtually all cells or Ad5-CMV *lacZ* as a control. Consistent with previous reports, Ad5-CB*CFTR*, but not Ad5-CMV *lacZ*, corrected defective CF cAMP-mediated Cl^- secretion. Basal Na^+ transport rates (basal I_{eq}) in CF airway epithelial sheets (-78.5 ± 9.8 $\mu A/cm^2$) were reduced to levels measured in normal epithelial sheets (-30.0 ± 2.0 $\mu A/cm^2$) by Ad5-CB*CFTR* (-36.9 ± 4.8 $\mu A/cm^2$), but not Ad5-CMV *lacZ* (-65.8 ± 6.1 $\mu A/cm^2$). Surprisingly, a significant reduction in ΔI_{eq} in response to ionomycin, a measure of Ca^{2+}-mediated Cl^- secretion, was observed in CFTR-expressing (corrected) CF epithelial sheets (-6.9 ± 11.8 $\mu A/cm^2$) when compared to uninfected CF epithelial sheets (-76.2 ± 15.1 $\mu A/cm^2$). Dose response effects of Ad5-CB*CFTR* on basal Na^+ transport rates and Ca^{2+}-mediated Cl^- secretion suggest that the mechanism of regulation of these two ion transport functions by CFTR may be different. In conclusion, efficient transduction of CFTR corrects hyperabsorption of Na^+ in primary CF airway epithelial cells and restores Ca^{2+}-mediated Cl^- secretion to levels observed in normal airway epithelial cells. Moreover, assessment of these ion transport abnormalities may represent important endpoints for testing the efficacy of gene therapy for cystic fibrosis. (*J. Clin. Invest.* 1995. 95:1377–1382). Key words: cystic fibrosis • airway epithelia • sodium transport • adenovirus-mediated gene transfer • CFTR

Introduction

Cystic fibrosis (CF),[1] the most common potentially lethal genetic disease in Caucasians, is caused by abnormal electrolyte transport in airway, sweat ductal, intestinal and pancreatic ductal epithelia (1, 2). The CF gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), has been shown to be a cAMP-activated Cl^- channel (3–5) and more recently to regulate the activity of other ion channels (6, 7). Complementation of the CF Cl^- permeability defect in vitro using adenovirus, retrovirus, vaccinia virus, and adeno-associated virus vectors strongly supports the notion that gene therapy for cystic fibrosis is feasible (7–11). These studies have also spurred current clinical safety and efficacy gene transfer trials in the nasal cavity and lower airways of CF patients (12, 13).

All of the efforts in gene transfer for CF thus far have focused on correction of abnormal cAMP-mediated regulation of Cl^- transport function (12). However, CF airway epithelia are also characterized by raised Na^+ transport (14, 15) and a raised alternative Ca^{2+}-regulated Cl^- secretory path (16–18). It has been speculated that abnormal Na^+ transport is central to the pathogenesis of CF airways disease (19), whereas the alternative Ca^{2+}-mediated Cl^- secretory path may be protective (18). Although not proven, both of these defects appear to reflect abnormal upregulation of channel expression/activity consequent to mutated CFTR (15, 16, 18).

In the present study, we tested whether introduction of wild-type CFTR into CF airway epithelia normalizes raised Na^+ absorption and raised Ca^{2+}-mediated Cl^- secretion in CF airway epithelia and consequently, whether these defects are potentially important endpoints of the efficacy of gene therapy.

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1. *Abbreviations used in this paper:* Δ, change; Ad5, adenovirus serotype 5; Ad5-CB*CFTR*, adenovirus serotype 5-based vector in which a CMV enhancer and β-actin promoter drive transcription of CFTR; Ad5-CMV *lacZ*, Ad5-based vector in which a CMV enhancer/promoter drives transcription of the β-galactosidase gene; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Cl_a, alternative chloride conductance; I_{eq}, equivalent short circuit current; LacZ, β-galatosidase; R_t, transepithelial resistance; V_t, transepithelial potential difference.

Methods

Cell culture. CF and normal nasal epithelial cells, isolated from surgical specimens by enzymatic digestion, were cultured as previously described (20). Cells were plated on permeable collagen substrates (surface area = 0.16 cm^2) at supraconfluent densities, maintained in serum-free medium (Ham's F12) supplemented with seven growth factors (20) until day 4 in culture, then switched to 50% Swiss 3T3-conditioned Dulbecco's modified Eagles medium with 4500 g/L glucose plus 2% bovine calf serum diluted in serum-free medium. In dose effect relationship experiments, cells were grown on collagen substrates (surface area = 0.071 cm^2) under air liquid interface conditions (21) in the absence of cholera toxin.

Adenoviruses. Adenovirus vectors used in this study are replication defective viruses based on the human adenovirus serotype 5 (Ad5). Their construction and the preparation of viral stocks was as previously described (22). In this study, Ad5-CMV *lacZ* and Ad5-CB*CFTR* with titers of up to 10^{11} pfu/ml were utilized.

Viral infections. Primary CF airway epithelial sheets were infected at confluence (culture day 3) overnight (~18 h) with 30 μl of either Ad5-CB*CFTR* or Ad5-CMV *lacZ* virus-containing medium at a multiplicity of infection (moi) of 10^4, or sham infected. Cells were then washed and maintained in 50% Swiss 3T3-conditioned medium for three additional days after infection. In the dose effect relationship experiments, cells were infected with 10 μl of Ad5-CB*CFTR* (in view of the smaller surface area) at the following mois: 0, 10^{-1}, 10^0, 10^1, 10^2, 10^3, and 10^4.

Bioelectric measurements. The transepithelial potential differences (V_t) and resistances (R_t) of all cultures were monitored (EVOM; World Precision Instruments, Inc., Sarasota, Florida) over days 4–6 in culture and the basal equivalent short circuit current (I_{eq}) calculated. To characterize the Cl^- transport function, cultures were mounted in a modified Ussing chamber interfaced to a current-voltage clamp (VCC600, Revision E; Physiologic Instruments, San Diego, CA) and superfused with Krebs-Ringer Bicarbonate solution, pH 7.4 at 37°C aerated with 95% O_2–5% CO_2. The sequential bioelectric responses to 10^{-4} M amiloride added to the luminal bath, luminal Cl^- replacement with 0 mM Cl^- containing Ringer (gluconate substituted for chloride), 10 μM forskolin, and 5 μM ionomycin were measured.

Western blots. Western Blots were performed as previously described (23). Samples were harvested by direct application of 35 μl urea buffer (67.5 mM Tris, pH 6.8; 7 M urea; 160 mM Dithiothreitol; 2% sodium dodecyl sulfate; 0.001% bromophenol blue) to the collagen substrate and stored at −70°C. Equal amounts of protein (50 μg/well) were loaded onto a 4–15% polyacrylamide gel and electrophoresis was performed at 110V for 60 min. Blotting onto polyvinylidene difluoride (PVDF) membranes (0.2 μm pore size; Bio Rad Labs., Hercules, CA) and immunodetection with anti-CFTR COOH terminus antibody was performed as previously described. T84 cell membranes were used as a marker of fully glycosylated CFTR.

Immunohistochemistry. Epithelial sheets on collagen membrane supports were detached from their polycarbonate supports using a scapel, embedded in OCT compound (Miles, Inc., West Haven, CT), and frozen at −70°C. Frozen sections (8-μm thickness) were cut on a Reichert Cryo-cut 1800 and stained for CFTR expression using a rabbit polyclonal anti-COOH terminus antibody (α1468, obtained from Dr. Jonathan Cohn, Duke University, Durham, NC) and a FITC-conjugated secondary antibody for immunofluorescent detection as previously described (24).

Statistics. All values are recorded as means±SE. Determination of statistical significance was performed using the student's *t* test. Significance was defined as $p < 0.05$. An analysis of variance (ANOVA) was used to detect statistical significance in the dose effect relationship experiments.

Results

Characterization of CFTR protein expression. As shown in Fig. 1 *A*, Western blot analysis revealed that CFTR protein was expressed in high levels in CF epithelial sheets infected with Ad5-CB*CFTR*, while CFTR was barely detectable in uninfected sheets or β-galactosidase (LacZ)-expressing epithelial sheets. Note that two bands were seen in whole cell lysates of CF cells expressing transduced CFTR: the fully glycosylated or mature form corresponding to a band at 180 kD and the core glycosylated protein at ~ 140 kD. T84 cell membranes, used as a marker of CFTR, exhibited a major band at 180 kD, which was expected since the fully glycosylated form would be primarily expressed in the membrane. CFTR protein could not be clearly detected in cultured primary normal airway epithelial cells (Fig. 1 *A*, lane *6*), although these cells exhibit normal Cl^- transport function. Shown in Fig. 1 *B* is immunohistochemistry for CFTR in corrected (Ad5-CB*CFTR*–infected) CF epithelial sheets. Note that staining is present in all cells facing the lumen in corrected sheets. Moreover, both apical and cytoplasmic staining patterns are present consistent with high level expression of CFTR. In contrast, no immunohistochemical staining for CFTR protein is detected in the Ad5-CB*CFTR*-infected CF epithelial sheets stained with secondary antibody alone (Fig. 1 *C*).

Characterization of basal Na^+ transport rates. Previous radioisotopic flux data have shown that basal I_{eq} equals net Na^+ absorption in cultured human airway epithelia (15). Because basal I_{eq} generally peaks on culture day 6, we monitored the basal I_{eq} in primary CF and normal airway cells daily from days 4–6 in culture. The expected pattern of a two- to threefold greater I_{eq} in uninfected CF cells as compared to uninfected normal cells was observed on days 5 and 6 in culture (Fig. 2 *A*). In contrast, the basal I_{eq} in corrected CF epithelial sheets was similar to the basal I_{eq} measured in the normal epithelial sheets, consistent with correction of Na^+ transport (Fig. 2 *B*). An approximately twofold reduction in the mean basal I_{eq} in CFTR-expressing CF epithelial sheets (corrected cells) was measured three days after infection (−36.9±4.8 $\mu A/cm^2$; Fig. 2 *B*) when compared to uninfected (−78.5±9.8 $\mu A/cm^2$; Fig. 2 *A*) or LacZ-expressing CF cells (−65.8±6.1 $\mu A/cm^2$; Fig. 2 *C*). The expression of LacZ or CFTR by Ad5 vectors did not alter the rate of Na^+ transport in normal airway epithelial cultures (compare Fig. 2, *A–C*).

Characterization of basal Cl^- transport function. We studied a series of CF and normal epithelial sheets in Ussing chambers to characterize their Cl^- secretory capacity. Shown in Fig. 3 *A* is the percent inhibition of I_{eq} by the Na^+ channel blocker, amiloride. Amiloride in airway epithelia both abolishes Na^+ transport and induces a driving force for Cl^- secretion (15). Thus, the percent inhibition of I_{eq} by amiloride reflects in part, the ability of the apical membrane to conduct transepithelial Cl^- ion flows. Amiloride inhibited basal I_{eq} in uninfected and LacZ-expressing CF cells ~ 90%, consistent with the absence of a significant basal Cl^- conductance. In contrast, a significantly lower percent inhibition of I_{eq} occurred post-amiloride in CFTR-expressing CF cells (61.8±4.8%), consistent, in part, with the expression of a basal Cl^- conductance.

The change in transepithelial potential difference with replacement of luminal Cl^- by gluconate ($\Delta V_{t\,Cl^-\,Free}$) reflects in part, the Cl^- permselectivity of the apical membrane. The small change (Δ) $V_{t\,Cl^-\,Free}$ measured in uninfected (−1.7±2.0 mV) and LacZ-expressing (−4.3±1.6 mV) CF cells is consistent with the absence of a significant apical membrane Cl^- conductance (Fig. 3 *B*). A significantly more negative $\Delta V_{t\,Cl^-\,Free}$ was detected in CFTR-expressing CF cells (−36.2±3.6 mV), sug-

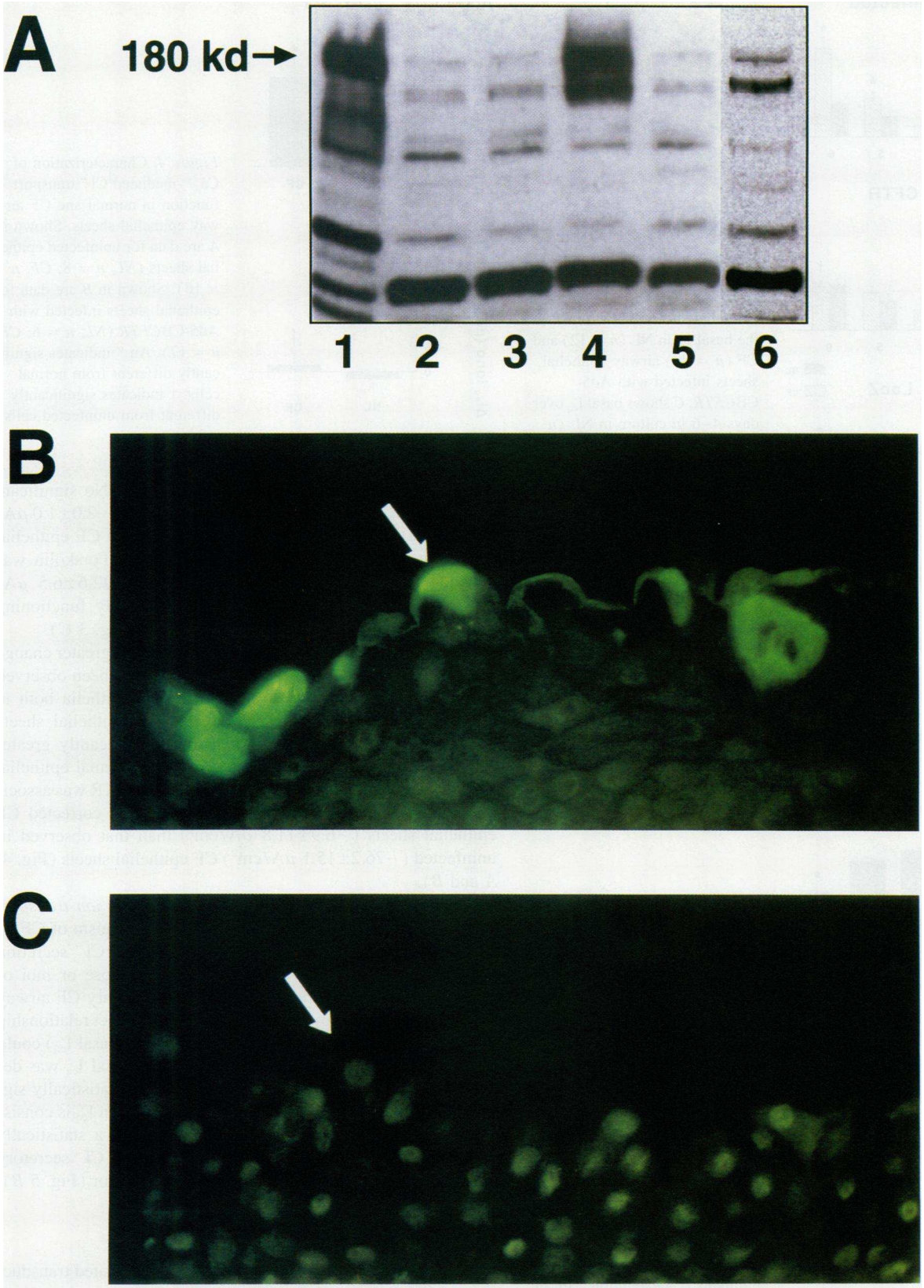

Figure 1. Characterization of CFTR expression in CF and normal epithelial sheets. (*A*) Western blot of CF airway cells from epithelial sheets infected with either Ad5-CB*CFTR* or Ad5-CMV *lacZ* using an anti-CFTR COOH terminus antibody. UNIF represents uninfected CF cells. LacZ represents CF epithelial cells three days after infection with Ad5-CMV *lacZ.* CFTR represents CF cells infected with Ad5-CB*CFTR* three days after infection. Lanes are as follows: *1,* T84; *2,* UNIF; *3,* LacZ; *4,* CFTR; and *5,* LacZ. Shown in lane *6* are uninfected cultured primary normal airway epithelial cells. *B* shows immunohistochemical detection of CFTR using an anti-COOH terminus polyclonal antibody (α1468) and a fluorescein-conjugated secondary antibody in an Ad5-CB*CFTR*-infected (corrected) CF epithelial sheet. The arrow points out a cell on the lumen expressing CFTR identified by the green fluorescent staining. Shown in *C* is a corrected CF epithelial sheet stained with secondary, but no primary antibody. Note the absence of CFTR staining on cells facing the lumen depicted by the arrow.

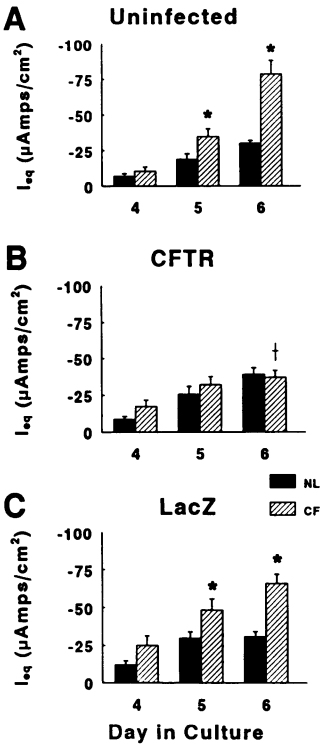

Figure 2. Characterization of basal Na^+ transport rates. *A* shows basal I_{eq} over days 4–6 in culture in uninfected NL (n = 10) and CF (n = 11) airway epithelial sheets. Shown in *B* is the basal I_{eq} in NL (n = 12) and CF (n = 20) airway epithelial sheets infected with Ad5-CB*CFTR*. *C* shows basal I_{eq} over days 4–6 in culture in NL (n = 11) and CF (n = 15) epithelial sheets infected with Ad5-CMV *lacZ*. * indicates significantly different from normal epithelial sheets. † indicates significantly different from uninfected epithelial sheets.

gesting the induction of a large apical membrane permeability in the corrected cells (Fig. 3 *B*).

Characterization of cAMP-mediated Cl^- transport function. The change in short circuit current induced by the cAMP-mediated agonist forskolin ($\Delta I_{eq\ Forskolin}$), was used to assess regulation of the apical membrane Cl^- conductance. No significant change in $\Delta I_{eq\ Forskolin}$ was detected in uninfected (-2.0 ± 1.0 $\mu A/cm^2$) and LacZ-expressing (-7.6 ± 2.0 $\mu A/cm^2$) CF epithelial sheets. In contrast, a significantly greater ΔI_{eq} Forskolin was detected in the CFTR-expressing CF cells (-22.6 ± 6.5 $\mu A/cm^2$), consistent with the induction of normally functioning CFTR Cl^- channels in the apical membrane (Fig. 3 *C*).

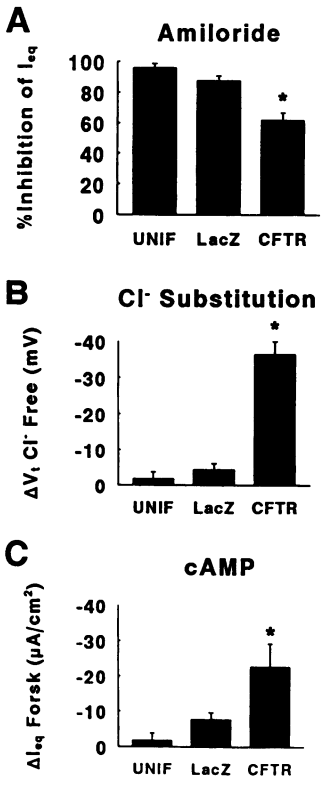

Figure 3. Characterization of basal and cAMP-mediated Cl^- transport function in CF airway epithelial sheets. *A* displays the percent inhibition of I_{eq} by amiloride. *B* shows the change in transepithelial voltage with luminal chloride substitution ($\Delta V_{t\ Cl^-\ Free}$). Shown in *C* is the change in I_{eq} in response to the cAMP-mediated agonist, forskolin ($\Delta I_{eq\ Forskolin}$). The responses displayed were sequential, ie., amiloride, added to the luminal bath, was followed by luminal Cl^- substitution (still in the presence of amiloride), and the subsequent addition of forskolin. * indicates significantly different from uninfected cells. UNIF, n = 5; LacZ, n = 9; CFTR, n = 10.

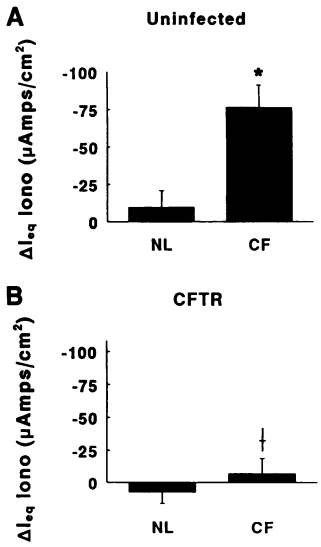

Figure 4. Characterization of Ca^{2+}-mediated Cl^- transport function in normal and CF airway epithelial sheets. Shown in *A* are data for uninfected epithelial sheets (*NL*, n = 8; *CF*, n = 16). Shown in *B* are data for epithelial sheets infected with Ad5-CB*CFTR* (*NL*, n = 6; *CF*, n = 17). An * indicates significantly different from normal cells. † indicates significantly different from uninfected cells.

Ca^{2+}-mediated Cl^- secretion. A consistently greater change in I_{eq} in response to Ca^{2+}-mediated agonists has been observed in CF epithelia compared to normal airway epithelia both in vitro and in vivo (16, 17). Uninfected CF epithelial sheets responded to ionomycin (5 μM) with a significantly greater $\Delta I_{eq\ ionomycin}$ than that measured in uninfected normal epithelial sheets (Fig. 4 *A*). Surprisingly, expression of CFTR was associated with a significantly smaller $\Delta I_{eq\ ionomycin}$ in corrected CF epithelial sheets (-6.9 ± 11.8 $\mu A/cm^2$) than that observed in uninfected (-76.2 ± 15.1 $\mu A/cm^2$) CF epithelial sheets (Fig. 4, *A* and *B*).

Dose effect relationship of Ad5-CBCFTR on ion transport function. To begin to address the issue of mechanism of CFTR regulation of Na^+ transport and Ca^{2+}-mediated Cl^- secretion by CFTR, we tested the effect of increasing dose or moi of Ad5-CB*CFTR* on ion transport function in primary CF airway epithelial cells. As shown in Fig. 5 *A*, no dose effect relationship of Ad5-CB*CFTR* on basal Na^+ transport rates (basal I_{eq}) could be demonstrated. A twofold reduction in the basal I_{eq} was detected at the highest moi of 10^4, although not statistically significant by an ANOVA. However, the reduction in I_{eq} is consistent with data presented in Fig. 2. In contrast, a statistically significant dose-related decline in Ca^{2+}-mediated Cl^- secretory current was detected with increasing moi of vector (Fig. 5 *B*).

Discussion

In the present study, we utilized adenovirus-mediated transduction of CFTR into CF airway epithelia to examine the effects of CFTR on the Na^+ absorptive pathway. We used a high moi in this study to ensure maximal gene transfer efficiency, because it was designed to test the concept that Na^+ transport is a correctable defect. The Western blot data (Fig. 1 *A*) presented indicate a high level expression of CFTR in corrected, but not

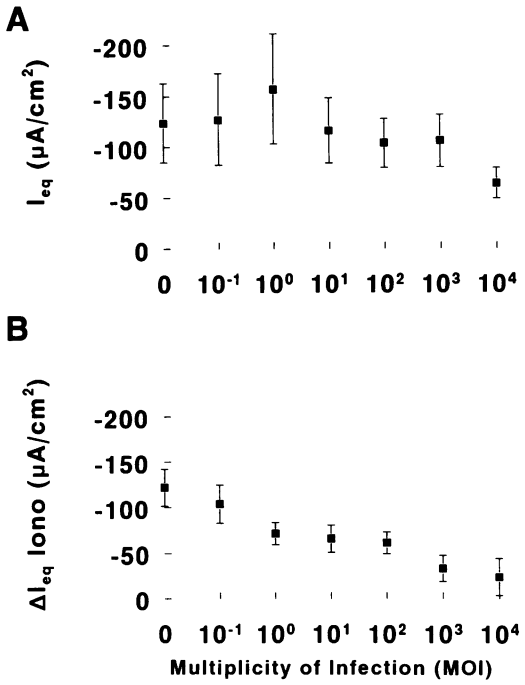

Figure 5. Dose Response of Ad5-CB*CFTR* on Na^+ transport and Ca^{2+}-mediated Cl^- secretion in primary CF epithelia. Shown in *A* is the effect of increasing doses (moi) on basal I_{eq} (Na^+ transport). *B* shows the effect of increasing moi on the change in current with ionomycin. n = 9–12 epithelial cell cultures for each dose of vector.

uninfected or LacZ-expressing CF tissues. Immunohistochemistry for CFTR (Fig. 1 *B*) not only confirms the western blot data, but also demonstrates that every cell facing the airway lumen has been transduced in Ad5-CB*CFTR*-infected CF epithelial sheets. Consistent with efficient transduction of CFTR, indices of Cl^- transport in CFTR-expressing tissues demonstrated restoration of basal and cAMP-mediated Cl^- secretion when compared to uninfected or LacZ-expressing CF epithelial sheets as reported by others (Fig. 3).

Using this strategy, we found that the CF Na^+ transport defect was corrected by introduction of wild-type CFTR. The rate of Na^+ transport (basal I_{eq}) was downregulated in CFTR-expressing CF epithelial sheets (Fig. 2 *B*) to rates similar to those of normal epithelial sheets. This effect appears to be specific to CFTR transduction and not vector exposure, because Ad5-CMV *lacZ* infection had no significant effect on basal I_{eq} (Fig. 2 *C*). Moreover, additional CFTR does not further downregulate Na^+ transport in normal cells.

Previous in vitro and in vivo studies suggest that the nonCFTR-mediated or alternative apical membrane Cl^- conductance (Cl_a) is increased in human CF airway epithelia when compared to normal airway epithelia (16, 17). Studies in the CF(−/−) mouse model demonstrated that Ca^{2+}-mediated Cl^- secretion occurs through this alternative pathway and that the Cl_a may be protective (18). In the present study, we found that adenovirus-mediated transduction of CFTR resulted in functional reduction of Ca^{2+}-mediated Cl^- secretion in CF epithelial sheets. This decrease in the Ca^{2+}-mediated Cl^- secretory response in corrected tissues is consistent with restoration of Cl_a pathway to normal levels.

Because the Na^+ channel subunits have only recently been cloned (25, 26) and Cl_a has not yet been cloned, it is difficult to explain the mechanism by which CFTR exerts effects on both Na^+ transport and Cl_a activity. To gain some insight into potential mechanisms, the dose effect relationships between vector moi and correction of Na^+ transport rates and Cl_a activity were examined (Fig. 5). Na^+ transport was not corrected until high mois were employed, consistent with a requirement for transduction of most cells in the monolayer (Fig. 1). This relationship suggests that correction of Na^+ transport is a linear function of CFTR transfer efficiency, and that little amplification of correction is achieved, contrasting with studies in which correction of as few as 6–10% of cells restored cAMP-mediated Cl^- transport to normal (27). The simplest hypothesis is that direct interactions of CFTR protein and Na^+ channel protein account for this phenomenon, and this interaction must occur in each transduced cell to normalize Na^+ channel function. This hypothesis is consistent with microelectrode and patch clamp data (15, 28), which indicate that the abnormally raised CF Na^+ transport rates reflect upregulation of apical membrane Na^+ entry, as manifested by an increase in Na^+ channel open probability (P_o). The ability of CFTR to regulate the functional activity of an independent ion channel, the outwardly rectifying chloride channel (ORCC), provides support for such a mechanism (6, 7).

In contrast, Cl_a function was reduced at low mois, implying that amplification of low efficiency CFTR transduction occurred. However, the decrease in Ca^{2+}-mediated Cl^- secretion (Cl_a) with increasing moi, as compared to an increase in cAMP-mediated Cl^- secretion (29), indicates that Cl^- movement through gap junctions (27) is not the mechanism responsible for the amplification of the Cl_a response. We speculate that this observation might be explained by an inverse relationship between expression of CFTR and Cl_a, as has been reported for CFTR and multidrug resistance (MDR) (30), or that other unknown factors regulating expression of Cl_a may be involved.

In summary, efficient expression of wild-type CFTR by Ad5-mediated gene transfer reduces raised Na^+ transport rates to levels observed in normal epithelial cell sheets. Hence, CFTR function is closely linked to regulation of Na^+ transport permitting, in theory, correction of both defective Cl^- secretion and Na^+ hyperabsorption by gene therapy. Correction of defective cAMP-mediated Cl^- transport and Na^+ hyperabsorption in this study was also associated with a reversal of the reciprocal relationship between functioning CFTR and Ca^{2+}-mediated Cl^- secretion. Because defects other than cAMP-mediated Cl^- transport may be important in pathogenesis of airways disease, these results suggest that future gene therapy efforts should develop protocols that measure all aspects of CF dysfunction and correction.

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