In vivo analysis of fluid transport in cystic fibrosis airway epithelia of bronchial xenografts

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Zhang, Yulong, James Yankaskas, James Wilson, and John F. Englehardt. In vivo analysis of fluid transport in cystic fibrosis airway epithelia of bronchial xenografts. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C1326–C1335, 1996.—An in vivo human bronchial xenograft model system was used to simultaneously analyze electrolyte and fluid transport defects in fully differentiated human cystic fibrosis (CF) and non-CF proximal airways. CF airways demonstrated three discernible defects when compared with non-CF, including *1*) a lack of adenosine 3′,5′-cylic monophosphate (cAMP)-inducible Cl^- secretion, *2*) a fourfold higher basal fluid absorption rate, and *3*) an altered regulation of fluid absorption in response to amiloride-stimulated changes in Na^+ transport. A unique finding in this study demonstrated that treatment of epithelia with amiloride led to a greater than threefold decrease in the rate of fluid absorption in CF tissues as contrasted to a greater than threefold increase in the rate of fluid absorption in non-CF tissues. The removal of apical Na^+ from amiloride-treated non-CF xenografts was capable of ablating this amiloride-induced increase in fluid absorption. In light of the recent interactions demonstrated between CF transmembrane conductance regulator (CFTR) and the rat epithelial, amiloride-sensitive Na^+ channel, these findings implicate additional complexities between the Na^+ conductance pathways and fluid transport in normal and CF proximal airways. Such findings suggest that CFTR may also regulate amiloride-insensitive Na^+ channels.

amiloride; insensitive; sodium; channels

CYSTIC FIBROSIS (CF) is characterized by defective regulation of chloride transport due to defects in the gene that encodes the CF transmembrane conductance regulator (CFTR; 4, 9, 13). Chronic lung infections, bronchiectasis, and respiratory failure are the primary causes of mortality in CF and are believed to be the result of abnormally thick mucus secretions that lead to obstructive lung disease and impaired mucociliary clearance of bacterial pathogens (1, 19). Mucociliary clearance in the lung is dependent on tightly regulated processes that control the extent of mucus secretion as well as the regulation of fluid transport (15). Both fluid and mucus secretions originate from two distinctly separate sites in the airways, including surface airway epithelia and submucosal glands (14). Localization studies defining the cell types that express CFTR in the lung have demonstrated that both bronchiolar airway epithelia and submucosal glands express significant levels of CFTR within specific subpopulations of cells (6, 8). Despite the advances in our understanding of CFTR function in the lung, the mechanisms by which defects in CFTR may lead to altered regulation of fluid transport in the airways have only begun to be unraveled. Current hypotheses suggest that defects in CFTR render airway epithelium less permeable to Cl^-, leading to altered electrolyte transport properties which in turn influence hydration of the surface epithelial fluid layer (1). This layer of fluid (called *sol*) is approximately 5 μm thick and provides a medium in which cilia can beat with only their tips contacting the viscoelastic gel coating of the airways (15). The fluid volume of this sol layer must be finely regulated, since small changes in the thickness of this layer will affect the efficiency with which cilia can move cellular debris and mucus up the airways. The mechanisms by which hydration of this sol layer is regulated are unknown but, in the proximal airways, could involve both airway epithelial cells as well as submucosal glands.

Electrolyte transport defects in CF have been extensively characterized in nasal epithelia both in vitro and in vivo (4, 9, 12, 13). Such in vivo studies have demonstrated that CF nasal epithelia have increased levels of amiloride-sensitive Na^+ conductance and are defective in a Cl^- secretory response to adenosine 3′,5′-cyclic monophosphate (cAMP) stimulatory agents. Direct characterization of fluid transport processes in the airway (in vivo) are lacking. However, correlation of electrolyte and fluid transport using in vitro polarized nasal monolayers have supported the notion that absorptive Na^+ conductance is a primary driving force of fluid absorption in airway epithelium, whereas stimulation of Cl^- secretion drives fluid secretion (11, 17). Additionally, in vivo studies of potential difference (PD) in nasal epithelium of CF patients have demonstrated increased amiloride-sensitive Na^+ conductance and a lack of cAMP agonist-stimulated Cl^- secretion (4, 17). With the recent cloning of the rat epithelial, amiloride-sensitive Na^+ channel (rENaC), pivotal experiments by Stutts and colleages (18) have demonstrated that functional CFTR inhibits Na^+ absorption through this amiloride-sensitive pathway. Together these in vitro and in vivo findings suggest a mechanism by which increased fluid absorption in CF airway epithelia leads to dehydration of mucus and impaired mucociliary clearance. However, in vivo confirmation of these proposed mechanisms of fluid balance in the airways has remained untested, due to the lack of adequate animal models that allow for the functional measurement of both electrolyte and fluid transport in a biological setting relevant to the human airway. Although in vitro nasal epithelial monolayers have proven useful in testing hypotheses that correlated fluid and electrolyte transport (11, 17), alterations in the differentiated

state of cells grown in culture may not accurately reflect in vivo regulatory processes involved in hydration of the sol layer.

This report describes an in vivo animal model of fully differentiated CF and non-CF human proximal airways that has been used to study the physiological relationship among Na^+, Cl^-, and fluid transport in airway epithelia. The ability to simultaneously correlate electrolyte transport, as measured by transepithelial PD, and fluid transport rates has allowed us to assess the contribution of Na^+ and Cl^- movement in the regulation of fluid transport across the airway epithelia. Comparison of normal airway epithelia with those defective in CFTR function has led to a better understanding of how epithelial fluid transport regulation may be altered in CF. Such studies have suggested that amiloride-insensitive Na^+ channels may also play a predominant role in fluid absorption in the proximal airway. Furthermore, this amiloride-insensitive pathway also appears to be defectively regulated in CF airway epithelium and implicates regulatory interactions of CFTR with an amiloride-insensitive Na^+ channel. These findings may ultimately lead to a more physiological assessment of primary defects in fluid transport within CF airway epithelium and provide a mechanistic basis for pharmacological intervention.

MATERIALS AND METHODS

Generation of CF and non-CF human bronchial xenografts. Human bronchial xenografts were generated from proximal bronchial tissue harvested from donor (non-CF) and recipient (CF) lungs at the time of lung transplantation. Primary cultures were prepared according to a previous protocol (7) with modification to include high antibiotics (in μg/ml: 80 tobramycin, 100 ceftazidime, 100 imipenem/cilastatin, 2.5 amphotericin B) within the culture medium. Human bronchial xenografts were generated from donor rat (male Fisher 344 rats; 300–350 g) tracheas denuded by three rounds of freeze thawing, followed by rinsing of the lumen with Eagle's minimum essential medium (7). Tracheas were ligated to tubing at both ends after seeding of $2–4 \times 10^6$ primary bronchial epithelial cells in 30 μl of hormonally defined medium. Two xenografts (one non-CF and one CF) were implanted subcutaneously in the flanks of male *nu/nu* BALB/c mice and flushed weekly with Ham's F-12 to remove excess mucus and other secreted proteins. By *week 3–4,* grafts developed a fully differentiated mucociliary epithelium. Grafts were analyzed for PD and fluid transport at 4–5 wk and harvested for morphological analysis as previously described (7).

Measurement of electrolyte transport by PD. At *week 4–5,* xenografts were analyzed for electrolyte transport using an electrode perfusion apparatus connected to one end of the xenografts (see Fig. 2, fluid apparatus at *point 1* detached). Electrodes were generated using 21-gauge butterfly needles filled with 1 M KCl in 4% agar. Transepithelial PDs were generated by introducing the positive agar electrodes into the luminal tubing of one end of the xenograft and the negative agar electrode subcutaneously in the back of the mouse. Electrodes were linked to a voltmeter through a set of calomel electrodes within a saturated KCl solution. Only agar electrodes that gave a PD of <0.2 mV after subcutaneous implantation of both electrodes were used for analysis. PD was measured with a sequence of perfused buffers as follows: *1*) *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) phosphate-buffered Ringer solution (HPBR) containing (in mM) 10 HEPES (pH 7.4), 145 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 Ca-gluconate, 2.4 K_2HPO_4, and 0.4 KH_2PO_4; *2*) HPBR with 100 μM amiloride; *3*) HPBR, 100 μM amiloride, Cl^- free (using gluconate in place of Cl^-); *4*) HPBR, 100 μM amiloride, Cl^- free, 200 μM 8-(4-chlorophenythio)cAMP (CPT-cAMP), 10 μM forskolin; and *5*) HPBR. Recordings were taken by means of a chart recorder and/or computer-assisted recording of millivolts every 5 s.

Fluid transport in CF and non-CF human bronchial xenografts. Fluid transport was measured from the bronchial xenografts by the attachment of 0.38 mm (ID) polypropylene tubing connected to one exiting port of the xenograft's tubing. The other end was held closed after instillation of buffer (Fig. 2, PD apparatus detached at *point 2*). Additionally, experiments were performed under conditions that simultaneously assessed fluid transport and transepithelial PD (see Fig. 2, both *points 1* and *2* attached). The volume of fluid movement through the capillary tubing was correlated with the volume displaced by measuring the distance a colored mineral oil/aqueous interface traveled over time. The entire fluid apparatus was filled with fluid to prevent any capillary effects on measurements. Fluid rates were typically measured between *minute 5* and *minute 15* after perfusion with buffers and gave between 5 and 10 points on which to generate a line. The slope of each line (correlation coefficient $r > 0.95$) directly correlated with the rate of fluid transport. The perfusion buffers used for fluid transport measurements were identical to those described for assessment of transepithelial PD. Additionally, fluid transport experiments were performed with non-CF xenografts in Na^+-depleted buffers, using the following perfusion sequence: *1*) HPBR (145 mM NaCl), *2*) HPBR (145 mM NaCl) with 100 μM amiloride, and *3*) HPBR (145 mM *N*-methyl-D-glucamine in place of NaCl) with 100 μM amiloride. These Na^+-depletion experiments were used to delineate the predominant conductance pathway responsible for amiloride-stimulated increases in fluid adsorption.

RESULTS

In vivo *transepithelial potential difference in CF and non-CF human bronchial xenografts.* Human primary bronchial epithelial cultures from matched CF and non-CF tissues harvested from recipient and donor lungs at the time of lung transplantation were grafted into denuded rat tracheas and implanted subcutaneously in the flanks of athymic mice. Four weeks posttransplantation a fully differentiated human bronchial epithelium developed that contained basal cells, goblet cells, intermediate cells, and ciliated cells (Fig. 1). Additionally, the distribution of various epithelial cell types, including basal, intermediate, goblet, and ciliated cells, was indistinguishable in CF and non-CF bronchial xenografts, suggesting that the culturing of bronchial epithelial progenitor cells was capable of removing secondary effects that lead to goblet cell hyperplasia in CF airway epithelium in vivo (compare Fig. 1, *A* and *B*). Previous studies have demonstrated that the pattern of cytokeratin-14, cytokeratin-18, and CFTR protein localization in xenografts gives an identical distribution to that of human proximal airway (7).

Between 4–5 wk posttransplantation, fully differentiated human bronchial CF and non-CF xenografts were analyzed for electrolyte transport, using an in

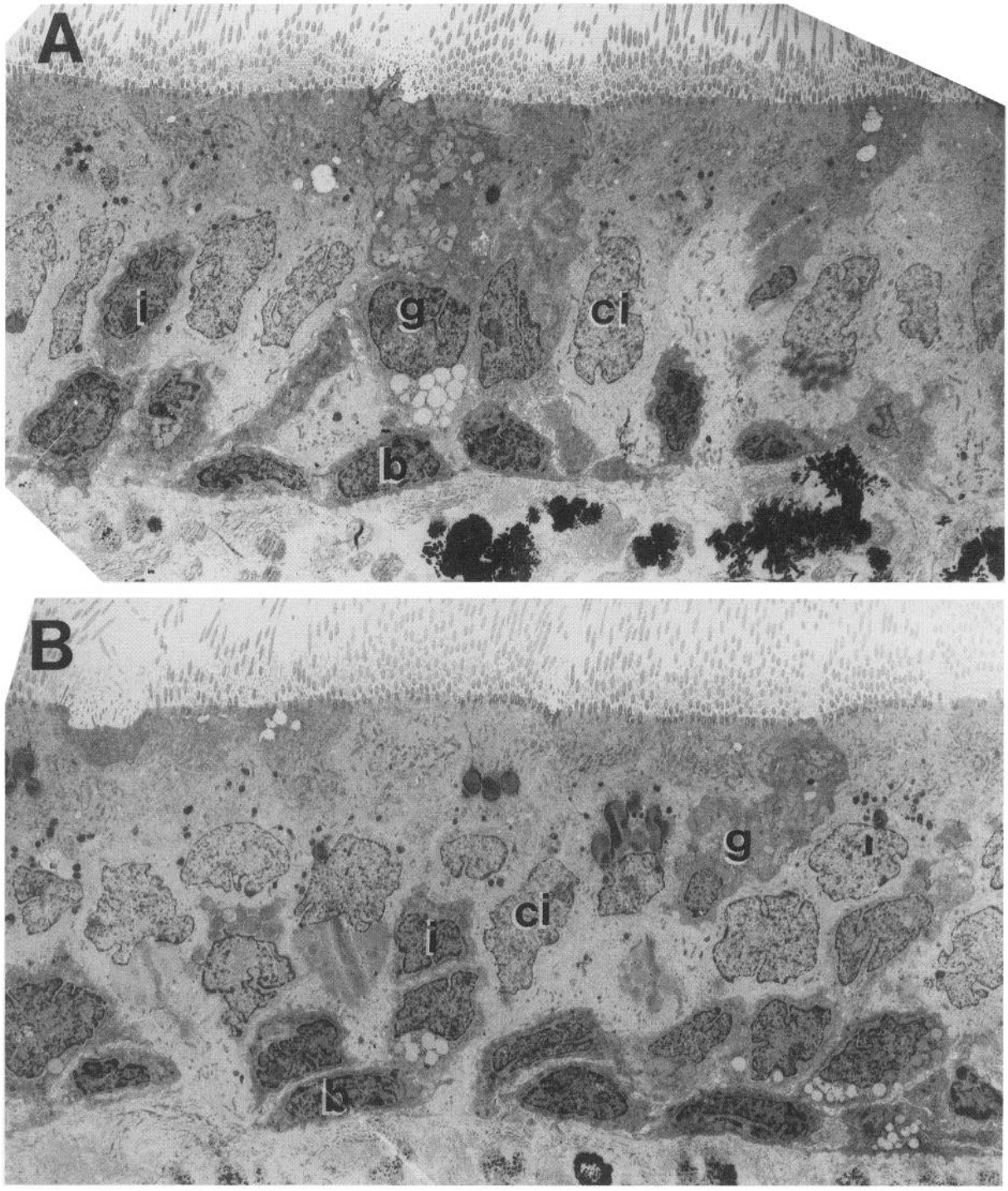

Fig. 1. Differentiated epithelium of CF and non-CF bronchial xenografts. Denuded rat tracheas seeded with 2–4 × 10^6 primary CF (*A*) and non-CF (*B*) bronchial epithelium and harvested 4 wk posttransplantation. b, Basal cells; i, intermediate cell; c, ciliated cell; g, goblet cell.

vivo PD perfusion apparatus (Fig. 2). Analyses were performed using two methods: *1*) steady perfusion with buffers at a rate of 150 μl/min (fluid measurement apparatus detached, see detachment *point 1* in Fig. 2) and *2*) simultaneous PD and fluid transport measurements in the absence of buffer perfusion (fluid measurement apparatus attached, see detachment *point 1* in Fig. 2). The measurement of PD in the absence of buffer perfusion caused significant drift in transepithelial PD. Hence, the results summarized in this report are the mean of PDs calculated from measurements performed under continuous buffer perfusion. The results summarize xenograft data derived from six independent tissue samples of human non-CF and CF bronchus. Genotypes for CF tissues included 1-ΔF508/ΔF508, 1-ΔF508/G542X, 2-ΔF508/unknown, 1-R553X/unknown, and

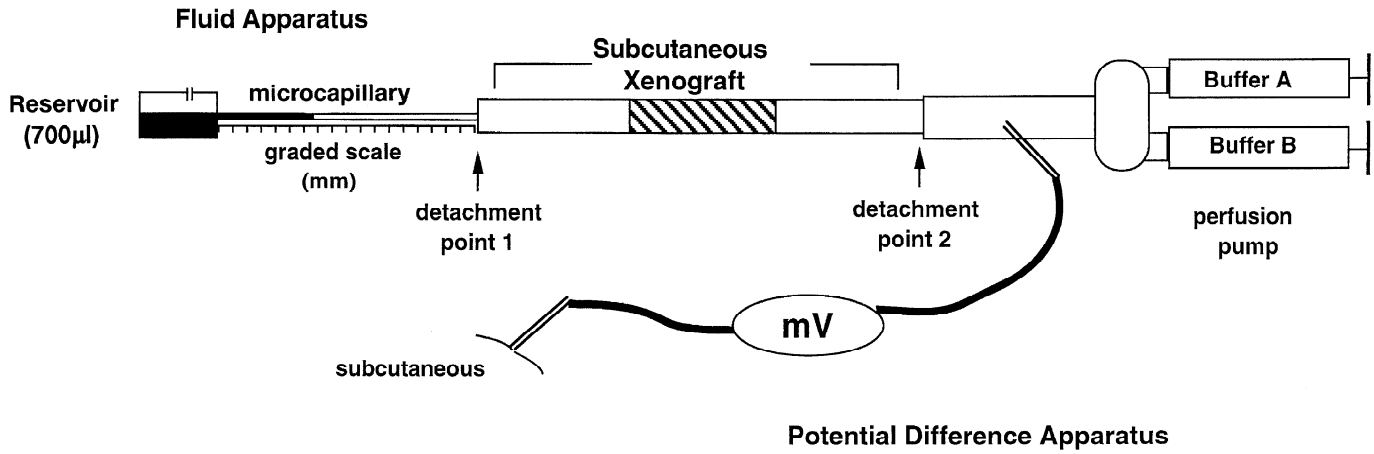

Fig. 2. In vivo transepithelial potential difference (PD) and fluid transport measuring apparatuses. Human bronchial xenografts were ligated to flexible plastic tubing and analyzed for transepithelial PD and fluid transport, using the diagrammed apparatuses. PD and fluid transport measurements can be analyzed simultaneously in the same graft (both *point 1* and *2* attached). Alternatively, PD and fluid measurements may be measured alone by detachment of *point 1* and *point 2*, respectively. Hatched area represents tracheal tissue.

1-G542X/G551D. A total of one to two independent xenografts was analyzed for each tissue sample to give a total number of independent experimental points of $n > 10$.

Transepithelial PD of non-CF epithelia demonstrated the characteristic profile that has been previously described with nasal airway epithelium both in vitro and in vivo (11, 12, 15) in response to amiloride-, Cl^--free, and cAMP-containing buffers (as described in MATERIALS AND METHODS). A typical transepithelial PD profile from a non-CF bronchial xenograft is shown in Fig. 3*A*. The results, summarized in Table 1, demonstrate the following findings: *1*) a baseline PD in HPBR (145 mM Cl^-) of -7.7 ± 1.7 mV (lumen negative), *2*) a change in PD to -3.1 ± 1.4 mV following the addition of amiloride (representing a $\Delta = +4.6$ mV), *3*) a change in PD to -13 ± 3.1 mV following perfusion with Cl^--free buffer in the presence of amiloride (representing a $\Delta = -9.8$ mV), and *4*) a change in PD to -22 ± 3.8 mV following the addition of CPT-cAMP-forskolin in Cl^- free, amiloride-containing buffers (representing a $\Delta = -9.3$ mV). All alterations in PD produced by

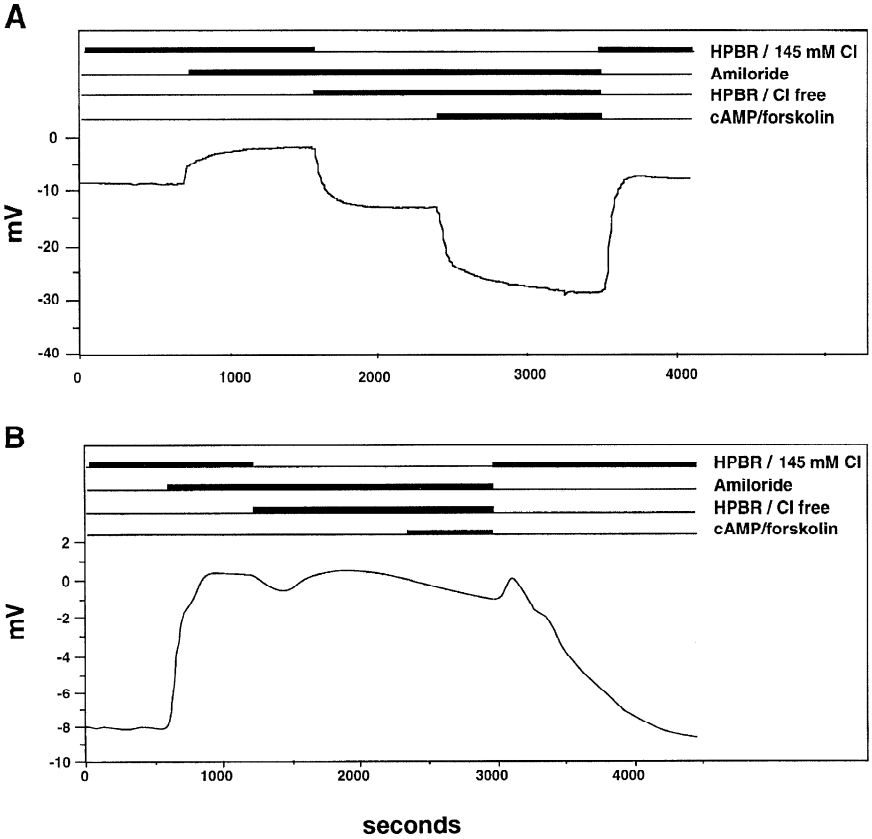

Fig. 3. Transepithelial PD measurements in CF and non-CF bronchial xenografts. Human bronchial non-CF (*A*) and CF (*B*) xenografts 4 wk posttransplantation were analyzed for transepithelial PD using the following sequence of continuous buffer perfusion (fluid apparatus detached): *1*) HEPES phosphate-buffered Ringer solution (HPBR) (145 mM Cl^-); *2*) HPBR (145 mM Cl^-), 100 µM amiloride; *3*) HPBR (0 mM Cl^-), 100 µM amiloride; *4*) HPBR (Cl^- free), 100 µM amiloride, 200 µM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP), 10 µM forskolin; *5*) HPBR (145 mM Cl^-).

Table 1. *Transepithelial potential difference in CF and non-CF bronchial xenografts*

	Transepithelial PD, mV				
	HPBR	Amil	Cl^- free	cAMP	HPBR
Non-CF	-7.7 ± 1.7	-3.1 ± 1.4†	$-13 + 3.1$†	-22 ± 3.8†	-8.6 ± 2.1†
CF	-8.5 ± 2.1	-2.4 ± 1.5†	-0.01 ± 0.74	-0.26 ± 0.7	-5.5 ± 1.3†
P value*	NS	NS	<0.001	<0.001	NS

Values are means ± SE of at least 10 independent measurements. Sequence of buffer perfusion was as follows: *1*) HPBR (145 mM Cl^-); *2*) Amil [HPBR (145 mM Cl), 100 μM amiloride]; *3*) Cl^- free [HPBR (0 mM Cl^-), 100 μM amiloride]; *4*) cAMP, [HPBR (0 mM Cl^-) + 100 μM amiloride, 200 μM CPT-cAMP, 10 μM forskolin]; *5*) HPBR (145 mM Cl^-). *Values for potential difference (PD) were compared for significant differences between CF and non-CF for each of the perfusion buffers by an unpaired Student's *t*-test ($n = 10$ different xenografts derived from 6 independent bronchial tissue samples); NS, no significant difference. †Values for PD were compared for significant differences within each CF and non-CF group after buffer changes using a paired Student's *t*-test (those marked demonstrate a significant change from the previous buffer, $P < 0.001$).

changes in amiloride, Cl^- free, and CPT-cAMP-forskolin-containing buffers were statistically significant when compared by a paired analysis using the Student's *t*-test ($P < 0.001$; $n = 10$ independent xenografts derived from 6 different tissue samples). The responses seen following the addition of amiloride are indicative of sodium-absorbing respiratory epithelia and provide a marker for comparison with fluid transport measurements that are described later in this report. With the contribution of Na^+ absorption blocked by the addition of amiloride, the perfusion of Cl^--free buffers, as well as the addition of CPT-cAMP-forskolin, produced changes typical of Cl^--secreting epithelium (i.e., transepithelial PD decreased toward lumen-negative values). Furthermore, adding 3-isobutyl-1-methylxanthine (500 μM) to CPT-cAMP-forskolin-stimulated non-CF epithelium produced no further change in PD (data not shown), suggesting that a maximal Cl^- secretory state has been achieved. Removal of CPT-cAMP-forskolin after stimulation, in the presence of low Cl^-, was incapable of reversing changes in PD over a time course of 15 min (data not shown), suggesting that once CFTR was activated, reversal of cAMP effects in the presence of a highly favorable electrochemical gradient was minimal. However, removal of both CPT-cAMP-forskolin and the electrochemical gradient produced by Cl^--free buffers produced a recovery to baseline PD in the presence of amiloride (data not shown).

To further confirm that changes seen after the addition of CPT-cAMP-forskolin were due to alteration in Cl^- permeability, xenografts were treated with mucosally applied bumetanide (a basolateral Na^+-K^+-$2Cl^-$ cotransport blocker) after CPT-cAMP-forskolin stimulation in Cl^--free, amiloride-containing buffer. Bumetanide produced a slow decrease in PD toward prestimulated levels over the course of 20 min (data not shown), suggesting that lowering the intracellular electrochemical gradient for Cl^- secretion lowered the PD response to CPT-cAMP-forskolin-containing buffers. This slow recovery in the presence of bumetanide is likely due to the relatively low permeability of this lipid-insoluble Na^+-K^+-$2Cl^-$ channel blocker to access the basolateral membrane when applied to the mucosal side of xenograft epithelial.

Comparison of the transepithelial PD profile of CF with non-CF bronchial xenografts was used to characterize difference in electrolyte transport (Table 1). All statistical comparisons were performed using an unpaired Student's *t*-test comparing xenografts derived from six independent CF and non-CF bronchial tissue samples. No significant difference was observed in the baseline PD of CF xenografts (-8.5 ± 2.1 mV) as compared with non-CF xenografts (-7.7 ± 1.7 mV). The addition of amiloride to mucosal buffer produced a change in transepithelial PD of two- to threefold (toward lumen positive) in both non-CF and CF epithelium. Although the mean ΔPD in the presence of amiloride was slightly higher in CF (6.1 mV ΔPD) compared with non-CF (4.6 mV ΔPD) xenografts, this change was not statistically significant ($n = 10$). In contrast to non-CF epithelia, CF tissues produced no significant changes in PD after exposure to Cl^- free or CPT-cAMP-forskolin-containing buffers, although there was a trend toward a more lumen-positive PD in most tissues analyzed after Cl^--free buffer perfusion. These results confirm that CF human bronchial xenograft epithelium is defective in the ability to induce Cl^- secretion under conditions known to stimulate CFTR.

As a measure of epithelial integrity after perfusion with various agonist-containing solutions, baseline transepithelial PD was measured in HPBR (145 mM Cl^-) following the completion of each experiment. No differences in the mean transepithelial PD in HPBR (145 mM Cl^-) were seen in either CF or non-CF tissues after each experiment (CF, -5.5 ± 1.3 mV; non-CF, -8.6 ± 2.1 mV). Additionally, analysis of Cl^- secretion in response to ATP (10^{-4} M) in the presence of amiloride, Cl^- free, and CPT-cAMP-forskolin-containing buffers demonstrated equivalent increases in PD (toward lumen negative) in both CF and non-CF xenografts (data not shown). These results confirm that the epithelial monolayer remained intact throughout the experiment and substantiate previous findings that the Ca^+-activated Cl^- channel remains unaltered in CF (10).

Fluid transport in CF and non-CF human bronchial xenografts. To begin to assess the contributions of Na^+ and Cl^- conductances on the net fluid transport in CF and non-CF xenograft airways, we measured the rate of fluid transport using microcapillary tubing (0.38 mm ID) attached to the end of xenografts in vivo. In these experiments, fluid transport was measured by the movement of a colored mineral oil/aqueous interface over time within a fluid-filled capillary tube in which the distal end was submerged in a colored mineral oil

reservoir (Fig. 2). Two methods were used for this analysis: *1*) fluid transport alone with one end of the xenograft held closed (PD apparatus detached; see detachment *point 2* in Fig. 2; for example of fluid rate measurements see Fig. 4), and *2*) simultaneous PD recording (no buffer perfusion) through one end of the xenograft with the microcapillary apparatus ligated to the opposite end of the xenograft (for example of recording, see Fig. 5). Both methods gave similar results and the data summarized in this report are the means of experiments performed using both techniques. Typically, an initial higher rate of fluid transport was seen within the first 1–5 min of measurements (Fig. 4*B*). A potential explanation for this finding was the time needed to equilibrate changes made after buffer perfusion. Analyses of differences in fluid transport rates between CF and non-CF tissues in response to amiloride, Cl^- free, and CPT-cAMP-forskolin were identical, regardless of whether the initial rate or the equilibrated rate was used for analysis. However, for purposes of this study, we have chosen to use only equilibrated rates of fluid transport that were typically made 5–15 min after the buffer changes. An example of differences in the initial rate and equilibrated rates as measured in human bronchial xenograft epithelium in the presence of HPBR (145 mM Cl^-) is shown in Fig. 4. Rates for fluid transport were determined from the slope of the line (distance vs. time) with correlation coefficients typically $r > 0.98$.

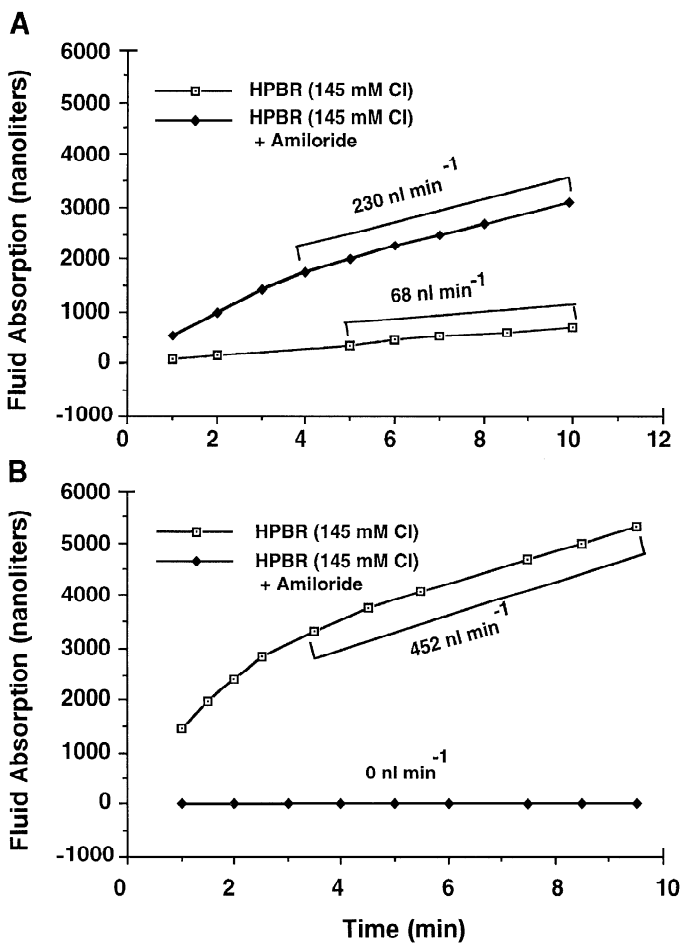

Fig. 4. Fluid transport measurements in CF and non-CF bronchial xenografts. Human bronchial non-CF (*A*) and CF (*B*) xenografts 4 wk posttransplantation were analyzed for fluid absorption in HPBR (145 mM Cl^-), followed by the addition of 100 μM amiloride to the same buffer. Rate of fluid absorption is indicated in brackets (slope of equilibrated fluid absorption line).

Data comparing the basal fluid transport rate in CF and non-CF xenografts demonstrate that these proximal airways are entirely absorptive under physiological conditions (Table 2). CF epithelium demonstrated a fourfold higher rate of fluid absorption (208 ± 32 nl·min^{-1}·cm^{-2}) as compared with non-CF epithelium (52 ± 13 nl·min^{-1}·cm^{-2}). These differences in basal fluid absorption rates between CF and non-CF were statistically significant ($P < 0.001$, $n = 12$) by an unpaired Student's *t*-test. For purposes of this study, the average surface area of the xenograft was calculated to be ~1 cm^2, based on the average length and luminal diameter.

To investigate the role of Na^+ conductance in modulating fluid absorption in non-CF and CF epithelia, we compared changes in the fluid absorption rates in response to amiloride. Treatment of CF epithelia with amiloride significantly decreased fluid absorption rates by a mean value of 141 nl·min^{-1}·cm^{-2} ($P < 0.003$, $n - 12$ by paired Student's *t*-test); all tissues showed at least a 50% decrease in fluid absorption upon treatment with amiloride, while several tissues demonstrated a complete lack of absorption in the presence of amiloride (Fig. 4*B*). This finding suggests that absorptive Na^+ conductance plays a predominant role in regulating fluid absorption in CF tissue. In contrast, treatment of non-CF tissues with amiloride produced a mean increase in fluid absorption rates of 130 nl·min^{-1}·cm^{-2} ($P < 0.001$, $n = 12$ by paired Student's *t*-test). Despite similar changes in transepithelial PD in response to amiloride between CF and non-CF tissues, fluid transport rates produced dramatically different responses representing a three- to fourfold decrease in fluid absorption in CF tissues as compared with a three- to fourfold increase in non-CF tissues. These differences between CF and non-CF amiloride-sensitive absorption rates were statistically different when compared by the unpaired Student's *t*-test ($P < 0.006$, $n = 12$). These findings suggest that the regulation of fluid transport in response to changes in amiloride-sensitive Na^+ permeability is altered in CF epithelium and implicate an alternative role of CFTR in regulating this process as it pertains to fluid transport.

To further characterize whether Na^+ conductance pathways were involved in this amiloride-stimulated increase in fluid absorption in non-CF xenografts, we performed additional experiments, analyzing fluid transport in Na^+-depleted, amiloride-containing buffers. Experiments summarizing the mean fluid absorption from four non-CF xenografts (2 independent tissue samples) are presented in Fig. 6. These xenografts demonstrated an increase in the basal rate of fluid absorption (58 ± 15 nl·min^{-1}·cm^{-2}) after the addition of amiloride (302 ± 74 nl·min^{-1}·cm^{-2}). This increase in fluid absorption was completely blocked by the

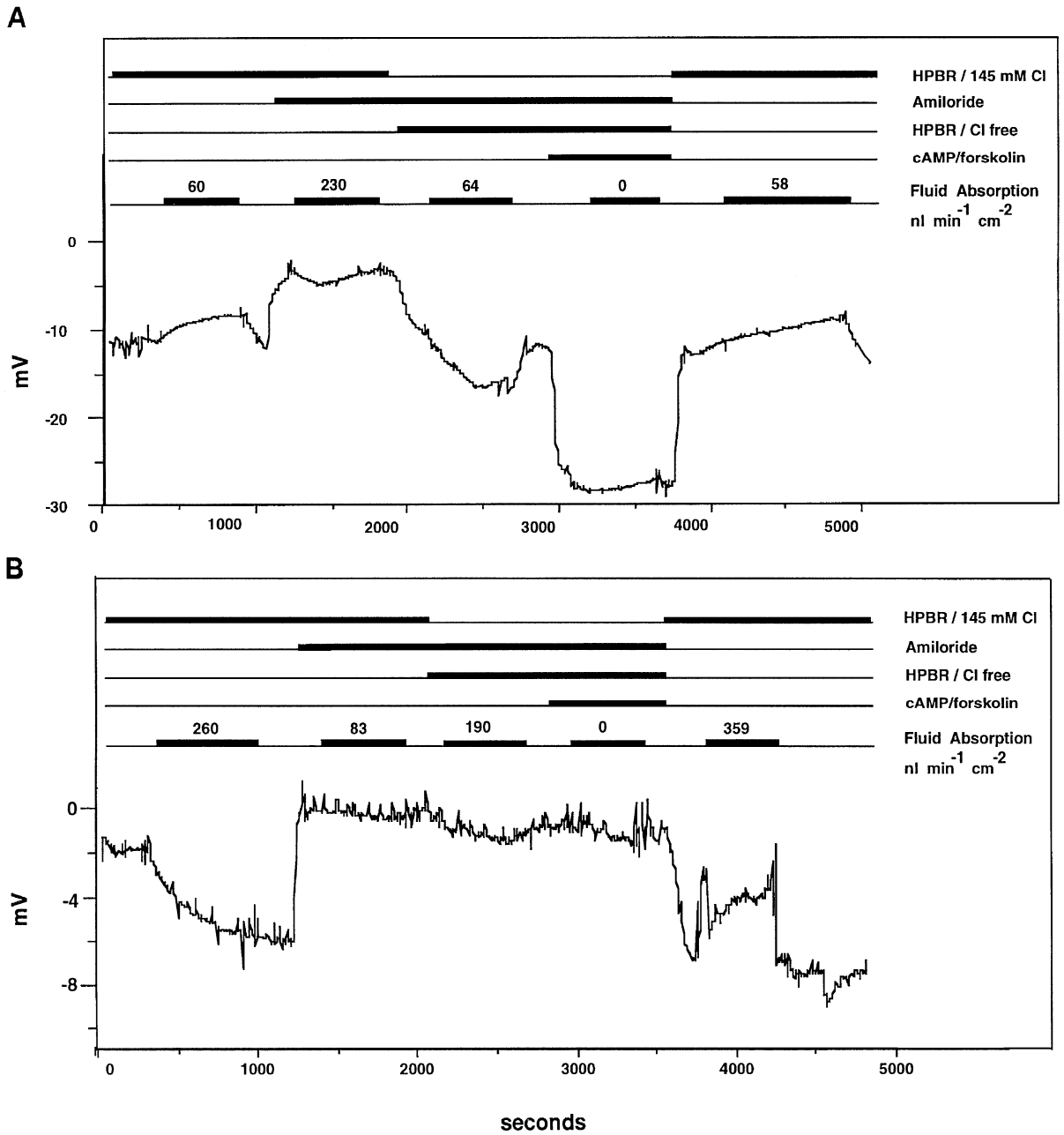

Fig. 5. Simultaneous fluid transport and transepithelial PD measurements in CF and non-CF bronchial xenografts. Human bronchial non-CF (*A*) and CF (*B*) xenografts 4 wk posttransplantation were analyzed for transepithelial PD using the following sequence of continuous buffer perfusion (fluid apparatus detached): *1*) HPBR (145 mM Cl^-); *2*) HPBR (145 mM Cl^-), 100 μM amiloride; *3*) HPBR (0 mM Cl^-), 100 μM amiloride; *4*) HPBR (0 mM Cl^-), 100 μM amiloride, 200 μM CPT-cAMP, 10 μM forskolin; *5*) HPBR (145 mM Cl^-). Once a baseline PD was achieved in each of the above buffers, buffer perfusion was stopped and the fluid measurement apparatus was attached for fluid transport recordings. Drift in PD was seen under conditions of static fluid perfusion. Equilibrated fluid absorption rates are indicated on graphs.

addition of amiloride-containing Na^+-free buffer (64 ± 35 nl·min^{-1}·cm^{-2}). These findings suggest that an amiloride-insensitive Na^+ channel is likely responsible for the altered fluid transport response to amiloride in non-CF tissues.

Analyses of changes in fluid absorption in response to treatment with Cl^- free and CPT-cAMP-forskolin-containing buffers were also performed to determine differences in the secretory capacity of CF and non-CF xenograft epithelia. Non-CF epithelia demonstrated a consistent decrease in the rate of fluid absorption in response to both of these treatments. Removal of Cl^- from the buffer in the presence of amiloride produced a 50% decrease in fluid absorption to a mean value of -96 ± 44 nl·min^{-1}·cm^{-2}. The response of several tissues to Cl^- free buffer was robust, producing a complete lack of all fluid absorption. Similarly, the addition of CPT-cAMP-forskolin further lowered fluid absorption to undetectable levels in all non-CF xenografts analyzed ($P < 0.001$, by paired Student's *t*-test).

Table 2. *Fluid absorption in CF and non-CF bronchial xenografts*

	Fluid Absorption, $nl \cdot min^{-1} \cdot cm^{-2}$				
	HPBR	Amil	Cl^- Free	cAMP	HPBR
Non-CF	52 ± 13	182 ± 24†	96 ± 44	<0.1 ± 0‡	28 ± 18
CF	208 ± 32	67 ± 25†	147 ± 87	16 ± 10	200 ± 108
P value*	<0.001	<0.006	NS	NS	NS

Values are means ± SE of at least 12 independent measurements. Sequence of buffer perfusion was as follows: *1*) HPBR (145 mM Cl^-); *2*) Amil [HPBR (145 mM Cl^-), 100 μM amiloride]; *3*) Cl^- free, [HPBR (0 mM Cl^-), 100 μM amiloride]; *4*) cAMP [HPBR (0 mM Cl^-) + 100 μM amiloride, 200 μM CPT-cAMP, 10 μM forskolin]; *5*) HPBR, (145 mM Cl^-). *Values for fluid absorption were compared for significant differences between CF and non-CF groups for each of the perfusion buffers by an unpaired Student's *t*-test ($n = 12$ different xenografts derived from 6 independent bronchial tissue sample); NS, no significant difference. †Values for fluid transport were compared for significant differences within each CF and non-CF group after addition of amiloride-containing buffer using a paired Student's *t*-test ($P < 0.003$). ‡No fluid absorption was seen with cAMP/forskolin, assumes a limit of sensitivity in measurements of 0.1 $nl \cdot min^{-1} \cdot cm^{-2}$.

Fluid secretion was never detected in any of the xenografts analyzed under Cl^- free or CPT-cAMP-forskolin-stimulated conditions. These findings suggest that fully differentiated airway epithelia may predominantly play an absorptive role in regulating fluid transport and that fluid secretion under the conditions analyzed in these experiments is negligible. Comparison of the transepithelial PD changes in response to Cl^- free and CPT-cAMP-forskolin-containing buffers suggests that in this model system the Cl^- secretory response is not sufficient to drive fluid secretion and may rather serve to counteract other mechanisms that drive fluid absorption in the epithelium (i.e., amiloride-insensitive Na^+ channels).

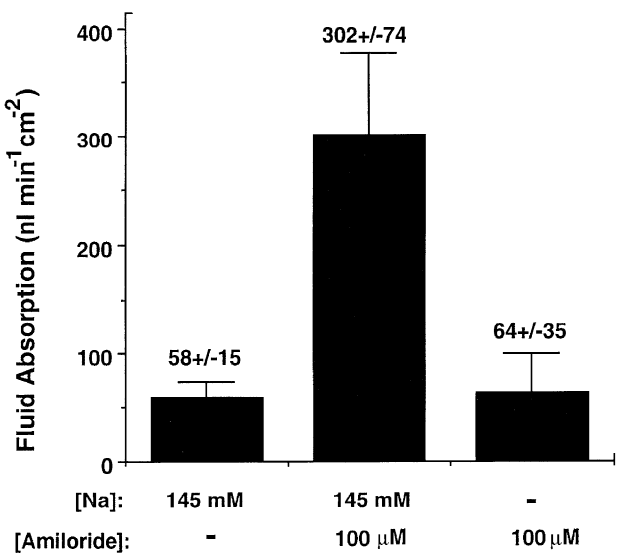

Fig. 6. Effect of Na^+ depletion on amiloride-stimulated fluid absorption in non-CF xenograft. Four xenografts derived from 2 independent tissue samples were analyzed for fluid absorption after perfusion of *1*) HPBR (145 mM NaCl); *2*) HPBR (145 mM NaCl), 100 μM amiloride; *3*) HPBR (145 mM *N*-methyl-D-glucamine in place of NaCl), 100 μM amiloride. Mean ± SE fluid absorption rate is plotted for each category.

CF tissues demonstrated a variable response in the presence of Cl^--free buffers in that some tissues increased fluid absorption rates while other did not. The mean steady-state fluid absorption rate of CF epithelia in Cl^- free buffers was indistinguishable from that in non-CF tissues. Treatment of CF epithelia with CPT-cAMP-forskolin lowered absorption rates in those tissues, which demonstrated an increase in fluid absorption after perfusion with Cl^--free buffer. In contrast, those tissues that remained unchanged in Cl^--free buffer showed no appreciable effect when CPT-cAMP-forskolin was added. In summary, although variable results comparing fluid transport rates between CF and non-CF xenografts were obtained with Cl^--free and cAMP-containing buffers, two consistent findings were observed, including *1*) a fourfold higher baseline fluid absorption rate in CF xenografts and *2*) a dysregulated Na^+-facilitated fluid absorption response after the addition of amiloride in CF as compared with non-CF xenografts.

DISCUSSION

Development of a human bronchial xenograft model of CF and non-CF proximal airway has allowed for the in vivo analysis of electrolyte transport as it relates to the regulation of fluid movement in a relevant biological setting. This model has several important advantages over previous in vitro and in vivo models of nasal epithelia (11, 17). The bronchial xenograft reconstitutes the cellular architecture of a fully differentiated mucociliary epithelium not achievable in vitro. Additionally, the confounding secondary consequences of infection and inflammation, such as goblet cell hyperplasia, are absent in CF xenografts (20).

In the present study, there were no significant differences in baseline (i.e., under physiological conditions) and amiloride-responsive transepithelial PDs between CF and non-CF xenografts. Previous reports have convincingly shown that both the baseline PD and amiloride-responsive PD are altered in nasal CF epithelia, suggesting that Na^+ absorption is elevated (4, 12). Several explanations may account for this discrepancy, including *1*) the contributions of disease (i.e., goblet hyperplasia) on the architecture and cellular constituents of the nasal epithelium, *2*) the presence of submucosal glands, and *3*) the differences in the normal cellular architecture of the nasal epithelium as compared with the bronchial epithelium. In support of the notion that electrolyte transport properties may be different in various airway epithelium systems are previous studies comparing baseline PDs in nasal, tracheal, and bronchial epithelium that demonstrate that more distal airways possess a less negative transepithelial PD (2, 3). Finally, our findings, which demonstrate an additional amiloride-insensitive Na^+ conductance responsible for fluid absorption in non-CF but not CF tissues, suggest that the baseline (i.e. physiological conditions) activity of this channel may also affect the resting transepithelial PD between CF and non-CF xenografts. This would lead to the hypothesis that this amiloride-insensitive Na^+ channel may

play a minor part in Na^+ conductance in nasal epithelium.

Analysis of PD responses to Cl^- free and CPT-cAMP-forskolin-containing buffers demonstrated a consistent finding in that non-CF xenograft epithelia possess Cl^- secretory responses, whereas CF xenograft epithelium did not. These results are consistent with a previous observation characterizing the involvement of CFTR in Cl^- secretory defects in CF nasal epithelium both in vitro and in vivo and demonstrate that the human bronchial xenograft epithelium retains the characteristics of both CF and non-CF airway epithelium with respect to CFTR-mediated Cl^- secretory function (5, 9, 13). Treatment of xenograft epithelia with bumetanide (100 μM), an inhibitor of the basolateral Na^+-K^+-$2Cl^-$ cotransporter, reversed stimulatory effects of CPT-cAMP-forskolin by lowering the intracellular pools of Cl^- and decreasing the electrochemical gradient for Cl^- across the apical membrane. These findings support the notion that cAMP stimulatory effects on PD are predominantly due to Cl^- secretion.

In an attempt to correlate changes in transepithelial PD with fluid movement in the conducting airway, we analyzed fluid transport within human bronchial xenografts by using a microcapillary apparatus that also provided a continuous record of transepithelial PD. Measurement of transepithelial PD in the absence of buffer perfusion produced substantial drift in the baseline PD (see Fig. 5). Presumably this is due to changes in local ion concentrations at the apical surface of epithelial cells, which in turn affect electrolyte transport. These fluctuations in PD suggest that the regulatory mechanisms of electrolyte transport are finely tuned in response to small changes in ion concentration. Our system, which has attempted to minimize these effects by using short measurement times and an excess of luminal fluid, provides an alternative method of analyzing fluid transport to that of previously studied in vitro models of polarized epithelial monolayers using microliter quantities of apical fluid overlaid with mineral oil and long assay times (<12 h) (17). Although the physiological relevance of each approach can be debated, each method is useful in assessing potential differences between the regulation of CF and non-CF conductance pathways that affect fluid transport.

Evaluation of fluid transport under physiological conditions demonstrated that the CF airway epithelium absorbs fluid (208 $nl \cdot min^{-1} \cdot cm^{-2}$) at a fourfold higher rate than non-CF epithelium (52 $nl \cdot min^{-1} \cdot cm^{-2}$). These results are strikingly similar to previous reports of nasal epithelial monolayers in vitro which demonstrate that a normal epithelium absorbs fluid at ~27 $nl \cdot min^{-1} \cdot cm^{-2}$ under basal conditions (11). Although others have also found that the basal fluid absorptive rate under physiological conditions is higher in CF compared with non-CF nasal epithelia, our findings differ in that this difference in fluid absorption was not represented as a higher lumen-negative PD (thought to be a marker of increased Na^+ absorption).

To investigate the regulatory influences of Na^+ transport on the pathological mechanisms of increased fluid absorption in CF, we evaluated amiloride-induced changes in fluid transport in CF and non-CF xenograft epithelium. Our findings demonstrated that reductions in Na^+ conductance led to a 3.1-fold ($P < 0.001$, $n = 12$) decrease in fluid absorption rates in CF epithelium. Unique to our study, however, was the finding that the inhibition of Na^+ conductance with amiloride in non-CF tissues led to a 3.5-fold increase ($P < 0.003$, $n = 12$) in the rate of fluid absorption by the airway epithelium. These findings suggest dysregulation between Na^+ and fluid transport in CF. Na^+ depletion in the presence of amiloride was capable of reversing the stimulated non-CF specific increases in fluid absorption and suggests that the interaction of CFTR with this amiloride-insensitive sodium channel may be defective in CF. The mechanism by which amiloride induced changes in the activity of an alternative amiloride-insensitive Na^+ channel remains unexplained but may be due to changes in the regulatory effects CFTR has on both amiloride-sensitive and -insensitive Na^+ channels. In support of this mechanism are recent studies that demonstrate an inhibitory effect of CFTR function on the amiloride-sensitive Na^+ channel (rENaC) (18). These findings might suggest that CFTR plays a dual role in the inhibition of amiloride-sensitive and the activation of amiloride-insensitive Na^+ channels. Recent evidence for abundant expression of an amiloride-insensitive, nonselective Na^+/K^+ channels in the rat tracheal airway (10) suggests that this channel may be a potential candidate involved in the altered Na^+-facilitated amiloride-insensitive fluid response seen in non-CF airways. Such findings, together with the recently demonstrated role of CFTR-mediated ATP transport in the regulation of the outwardly rectifying Cl^- channels (16), suggest that CFTR may interact within a complex of channels to regulate electrolyte and fluid transport in the airway.

The present study has attempted to evaluate the contributions of Na^+ and Cl^- transport to the regulation of fluid movement across fully differentiated human airway epithelium. Reconstitution of an in vivo airway with genetically defined bronchial epithelia has allowed us to study the dysregulation of these processes in CF. Our in vivo results have demonstrated that changes in amiloride-sensitive Na^+ transport across the apical membrane of respiratory epithelial cells produces a quantitatively significant difference in fluid absorption in CF compared with non-CF epithelium. Despite the lack of a clearly defined mechanism, our studies clearly indicate that defects in CFTR lead to altered regulation in fluid absorption through an amiloride-insensitive, Na^+-facilitated pathway. Although our findings support the therapeutic application of amiloride in increasing the hydration of mucus in CF by decreasing fluid absorption in the airways, the effect of amiloride on increasing fluid absorption in non-CF epithelium suggests that the traditional mechanisms by which this drug acts in CF may require reevaluation. Additionally, these findings, which suggest that

defects in CFTR may regulate intracellular processes involved in fluid movement across the airway epithelium, have implications for the efficiency of gene therapy approaches that may be required to target intracellular regulatory defects.

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