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Loss of Anion Transport without Increased Sodium Absorption Characterizes Newborn Porcine Cystic Fibrosis Airway Epithelia

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SUMMARY

Defective transepithelial electrolyte transport is thought to initiate cystic fibrosis (CF) lung disease. Yet, how loss of CFTR affects electrolyte transport remains uncertain. $CFTR^{-/-}$ pigs spontaneously develop lung disease resembling human CF. At birth, their airways exhibit a bacterial host defense defect, but are not inflamed. Therefore, we studied ion transport in newborn nasal and tracheal/bronchial epithelia in tissues, cultures, and in vivo. $CFTR^{-/-}$ epithelia showed markedly reduced Cl^- and HCO_3^- transport. However, in contrast to a widely held view, lack of CFTR did not increase transepithelial Na^+ or liquid absorption or reduce periciliary liquid depth. Like human CF, $CFTR^{-/-}$ pigs showed increased amiloride-sensitive voltage and current, but lack of apical Cl^- conductance caused the change, not increased Na^+ transport. These results indicate that CFTR provides the predominant transcellular pathway for Cl^- and HCO_3^- in porcine airway epithelia, and reduced anion permeability may initiate CF airway disease.

INTRODUCTION

Loss of cystic fibrosis transmembrane conductance regulator (CFTR) function causes CF (Davis, 2006; Quinton, 1999; Rowe et al., 2005; Welsh et al., 2001). Disease manifestations appear in many organs, but most morbidity and mortality currently arise from airway disease, where inflammation and infection destroy the lung. Understanding the pathogenesis of lung disease has been difficult, and there are many theories to explain how deficient CFTR function causes airway disease (Boucher, 2007; Davis, 2006; Quinton, 1999; Rowe et al., 2005; Verkman et al., 2003; Welsh et al., 2001; Wine, 1999). One factor impeding progress in identifying the events that initiate airway disease has been lack of an animal model that replicates features of the disease; mice with mutated *CFTR* genes do not develop gastrointestinal or lung disease typical of human CF (Grubb and Boucher, 1999). Therefore, we recently developed $CFTR^{-/-}$ pigs (hereafter referred to as CF pigs) (Rogers et al., 2008b). At birth, they manifest features typically observed in patients with CF, including pancreatic destruction, meconium ileus, early focal biliary cirrhosis, and microgallbladder (Meyerholz et al., 2010b). Within a few months of birth, CF pigs spontaneously develop lung disease with the hallmark features of CF including inflammation, infection, mucus accumulation, tissue remodeling, and airway obstruction (Stoltz et al., 2010).

Finding that CF pigs develop airway disease like that in humans provided an opportunity to explore very early events in the disease. We previously showed that within hours of birth, CF pigs have a reduced ability to eliminate bacteria that either enter the lung spontaneously or that are introduced experimentally (Stoltz et al., 2010). However, like newborn human babies with CF, CF pigs lack airway inflammation at birth. Those data indicate that impaired bacterial elimination is the pathogenic event that begins a cascade of inflammation, remodeling and pathology in CF lungs. Thus, these newborn animals provide an ideal model in which to evaluate ion transport processes because they possess the host defense defect, but they do not yet exhibit inflammation, tissue remodeling or other features of progressive CF. Hence, electrolyte transport defects can be attributed to loss of CFTR rather than to secondary manifestations of the disease.

Abnormal electrolyte transport across airway epithelia has frequently been hypothesized to cause the initial CF host defense defect (Boucher, 2007; Davis, 2006; Quinton, 1999; Rowe et al., 2005; Verkman et al., 2003; Welsh et al., 2001; Wine, 1999). In CF epithelia, loss of CFTR decreases airway Cl^- and HCO_3^- transport. This result is consistent with the anion channel activity of CFTR (Sheppard and Welsh, 1999). Some have also concluded that CFTR negatively regulates epithelial Na^+ channels (ENaC); hence *CFTR* mutations are proposed to eliminate that ENaC inhibition, increase Na^+ permeability, and cause Na^+ hyperabsorption, which is widely viewed as the initial event in CF lung disease pathogenesis (Boucher, 2007).

To understand how CF affects airway epithelial ion transport, we asked if loss of CFTR would disrupt transepithelial Cl^-, HCO_3^-, and Na^+ transport in CF pigs. We studied newborn animals to identify defects prior to the onset of inflammation. Knowledge of the extent to which these processes are disrupted is key to understanding CF airway disease and is important for developing mechanism-based treatments and preventions.

RESULTS

CF Pig Airways Lack cAMP-Stimulated Cl^- and HCO_3^- Transport

We measured the nasal and tracheal transepithelial voltage (Vt) in vivo in newborn pigs. Perfusion of the apical surface of epithelia with a Cl^--free solution and isoproterenol (to increase cellular cAMP levels) hyperpolarized Vt in non-CF pigs (Figures 1A and 1B) (Rogers et al., 2008b). In contrast, Vt failed to hyperpolarize in CF pigs. These data suggest a lack of cAMP-stimulated Cl^- permeability in CF.

When non-CF nasal, tracheal, and bronchial epithelia were excised or cultured as differentiated airway epithelia and studied in Ussing chambers, adding forskolin and isobutylmethylxanthine (IBMX) to elevate cellular cAMP levels increased absolute values of Vt (Figures 1C and 1D), short-circuit current (Isc) (Figures 1E and 1G), and transepithelial electrical conductance (Gt) (Figures 1F and 1H). Adding GlyH-101, which inhibits CFTR (Figure S1 available online) (Muanprasat et al., 2004), had the opposite effects (Figure 1I–1L). In contrast, CF epithelia failed to respond to either forskolin and IBMX or GlyH-101 (Figure 1C–1L). CFTR has a significant HCO_3^- conductance, and human non-CF airway epithelia transport HCO_3^- (Poulsen et al., 1994; Smith and Welsh, 1992). When we studied non-CF tracheal epithelia in Cl^--free bathing solution containing 25 mM HCO_3^-, forskolin and IBMX stimulated and then GlyH-101 inhibited Isc and Gt (Figures 1M and 1N), revealing electrically conductive HCO_3^- transport. CF epithelia lacked these responses.

These data indicate that porcine CF airway epithelia extending from nose to bronchi lack cAMP-stimulated Cl^- and HCO_3^- permeability. Our findings agree with studies of human airway epithelia, which have consistently demonstrated a loss of Cl^- and HCO_3^- permeability in CF airway epithelia (Knowles et al., 1983; Smith and Welsh, 1992; Standaert et al., 2004; Widdicombe et al., 1985). Moreover, our results indicate that in wild-type porcine airway epithelia, CFTR provides an important transepithelial pathway for Cl^- and HCO_3^-.

Vt Is Abnormal in CF Nasal, but Not Tracheal Epithelia In Vivo

The first indication of abnormal electrolyte transport in CF airways was the finding that nasal Vt was more electrically negative in CF than non-CF subjects and that amiloride produced a greater reduction in Vt ($\Delta Vt_{amiloride}$) in CF (Knowles et al., 1981). Those and additional observations led the authors to conclude that CF epithelia have increased Na^+ absorption that depletes periciliary liquid, which in turn impairs mucociliary clearance and initiates lung disease (Boucher, 2007; Donaldson and Boucher, 2007).

There is evidence that changes in Na^+ transport can affect the lung. For example, transgenic mice overexpressing the β subunit of the epithelial Na^+ channel (βENaC) had lung disease that shared some features with CF (Mall et al., 2004). Mutations have also been reported in human *ENaC* genes, and they may contribute to lung disease with some CF-like features. However, the *ENaC* mutations are associated with both decreases and increases in ENaC activity (Azad et al., 2009; Baker et al., 1998; Huber et al., 2010; Kerem et al., 1999; Schaedel et al., 1999; Sheridan et al., 2005). Thus, while alterations in Na^+ permeability can contribute to lung disease, those results do not indicate whether Na^+ absorption is increased, reduced, or unchanged in CF.

Therefore, we measured Vt and the response to amiloride in vivo in newborn pigs. In the nose, Vt and $\Delta Vt_{amiloride}$ were greater in CF than non-CF pigs (Figures 2A and 2C) (Rogers et al., 2008b). Remarkably, this was not the case in tracheal epithelia; Vt and $\Delta Vt_{amiloride}$ were similar in non-CF and CF pigs (Figures 2B and 2C).

Earlier studies showed that Vt and $\Delta Vt_{amiloride}$ are more negative in nasal and tracheal epithelia of CF patients than in non-CF controls (Davies et al., 2005; Knowles et al., 1981; Standaert et al., 2004). Our data in porcine nasal epithleia parallel those results. However, interestingly, when measurements were made in main bronchi and distal airways of children, Vt values were similar in CF and non-CF (Davies et al., 2005). Those results are like the data in porcine trachea. It seems that airway region and age, and perhaps inflammation and infection influence the activity of epithelial ion channels and thereby whether a Vt difference exists between CF and non-CF epithelia. It will also be important to study electrolyte transport in vivo, in excised tissue, and in cultures from older CF pigs as the disease progresses.

Absorptive Na^+ Fluxes Are Not Increased in Porcine CF Airway Epithelia

The difference in Vt between CF and non-CF nasal epithelia could relate to differences in Na^+ transport. Therefore, we directly examined Na^+ transport by measuring transepithelial $^{22}Na^+$ fluxes. We studied primary cultures of differentiated airway epithelia and used open-circuit conditions to mimic the in vivo situation. There were three main observations. First, in tracheal epithelia, unidirectional and net Na^+ fluxes did not differ between CF and non-CF epithelia (Figure 3A, Table S1). Adding amiloride decreased the unidirectional absorptive (apical to basolateral) and net Na^+ fluxes, indicating the importance of apical Na^+ channels for Na^+ absorption. Second, in nasal epithelia, Na^+ fluxes and the response to amiloride were also similar in CF and non-CF epithelia (Figure 3B). Third, nasal epithelia had greater unidirectional absorptive fluxes and net Na^+ absorption than tracheal epithelia (compare Figures 3A and 3B).

Like our data in pigs, in human nasal epithelia, $^{22}Na^+$ fluxes measured under open-circuit conditions revealed no difference between non-CF and CF (Willumsen and Boucher, 1991a, 1991b). Under short-circuited conditions, which differ from the in vivo situation, net $^{22}Na^+$ fluxes were reported to be either the same or increased in CF versus non-CF (Boucher et al., 1986; Knowles et al., 1983).

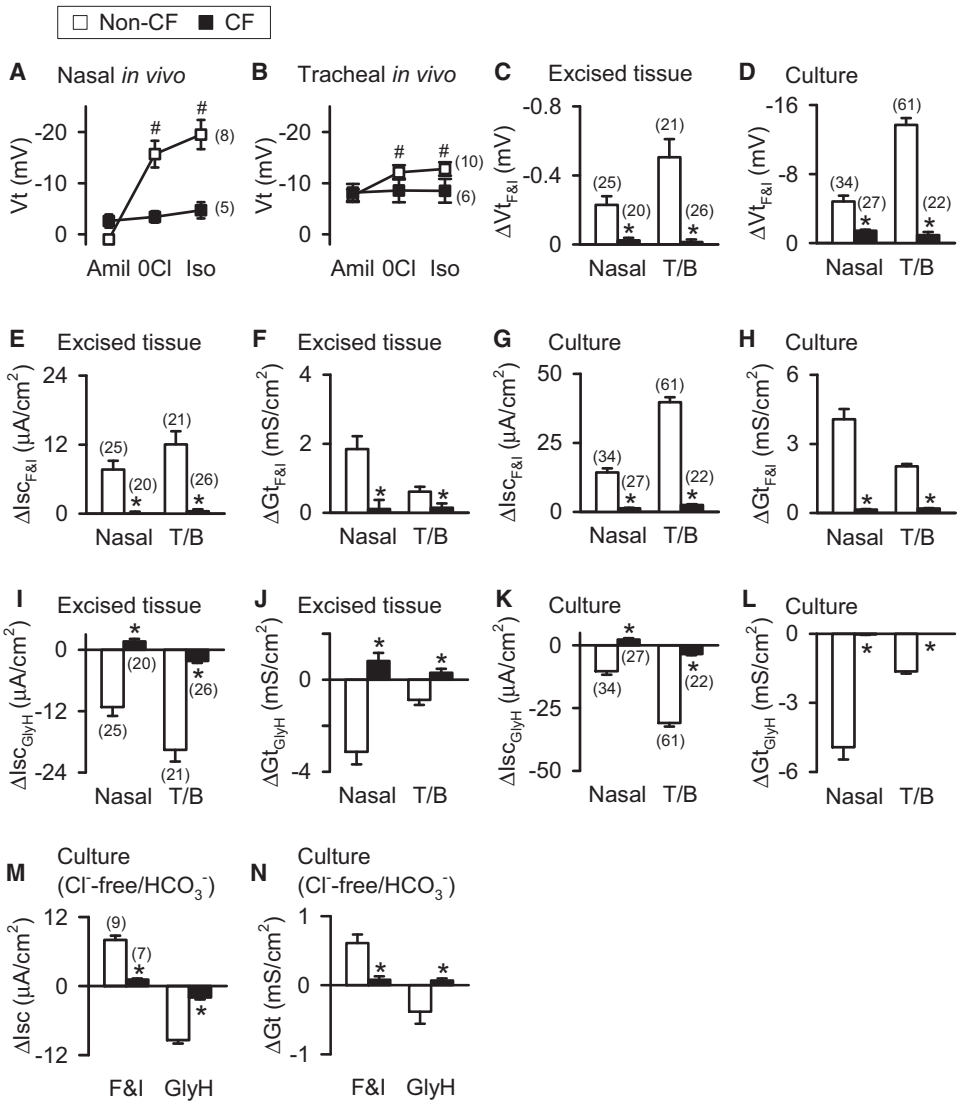

Figure 1. Loss of CFTR Decreases Anion Transport in CF Airway Epithelia

Data are means ± SE from newborn *CFTR*$^{+/+}$ (open symbols and bars) and *CFTR*$^{-/-}$ (closed symbols and bars) pigs. Amiloride (100 μM) was present on the apical surface in all cases. Numbers in parentheses indicate n, asterisk indicates $p < 0.05$ between CF and non-CF, and T/B indicates tracheal/bronchial.

(A and B) Vt measured in vivo in nasal and tracheal epithelia in the presence of amiloride (100 μM), during perfusion with a Cl^--free solution (0Cl) containing amiloride, and during perfusion with a Cl^--free solution containing isoproterenol (10 μM) and amiloride. Nasal epithelia include data from four non-CF and four CF pigs that were previously reported (Rogers et al., 2008b). #$p < 0.05$ compared to initial value.

(C–H) Change in Vt, Isc, and Gt induced by adding 10 μM forskolin and 100 μM IBMX ($\Delta Vt_{F\&I}$, $\Delta Isc_{F\&I}$, and $\Delta Gt_{F\&I}$) to excised and cultured nasal and tracheal/bronchial epithelia.

(I–L) Change in Isc (ΔIsc_{GlyH}) and Gt (ΔGt_{GlyH}) following addition of GlyH-101 (100 μM) to excised and cultured nasal and tracheal/bronchial epithelia.

(M and N) CFTR-mediated HCO_3^- transport in cultured tracheal epithelia. Solution was Cl^--free and contained 25 mM HCO_3^-. Data are ΔIsc and ΔGt following addition of forskolin and IBMX and GlyH-101.

See also Figure S1.

Liquid Absorption Is Not Increased in Porcine CF Epithelia

We also measured rates of transepithelial liquid absorption, which is driven by Na^+ absorption. Liquid absorption rates were greater in nasal than tracheal epithelia, consistent with the $^{22}Na^+$ fluxes (Figure 3C). However, CF epithelia did not absorb liquid at a greater rate than non-CF epithelia. In fact, in nasal epithelia, the absorption rate was less in CF than non-CF epithelia.

In studies of cultured human airway epithelia, the initial rate of liquid absorption has been reported to be increased (Matsui et al., 1998), similar (Van Goor et al., 2009), or reduced (Zabner et al., 1998) in CF compared to non-CF. The reason for the

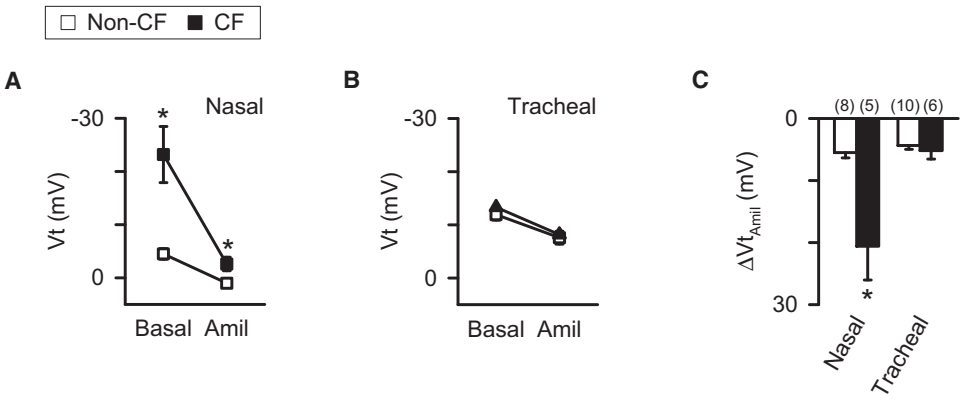

Figure 2. Vt In Vivo Is Abnormal in CF Nasal Epithelia, but Not Tracheal Epithelia

Data are mean ± SE from *CFTR*$^{+/+}$ (open symbols and bars) and *CFTR*$^{-/-}$ (closed symbols and bars) pigs.

(A and B) Effects of amiloride (100 μM) on nasal and tracheal Vt in vivo.

(C) Amiloride-sensitive change in Vt ($\Delta Vt_{amiloride}$) in vivo. *$p < 0.05$ compared to non-CF.

differences is uncertain, but might relate to variations in basal CFTR activity (Zabner et al., 1998).

The Depth of Periciliary Liquid Is Not Altered by Lack of CFTR

Transepithelial ion and H_2O movement contribute to the depth of liquid covering the airway surface. Twenty-four hours after adding liquid to the apical surface of cultured epithelia, the depth of periciliary liquid has been reported to be less in CF compared to non-CF epithelia (Matsui et al., 1998; Van Goor et al., 2009). A study that obtained bronchoscopic biopsies from patients with CF reported that although not statistically significant, there was a trend toward reduced periciliary liquid height in CF (Griesenbach et al., 2010). However, the authors noted that inflammation (most patients were experiencing a respiratory exacerbation) and the methods used (periciliary liquid height could not be measured in half the patients or over the majority of cells) limit the interpretation.

To test the hypothesis that loss of CFTR alters periciliary liquid depth in the absence of infection, inflammation, and tissue remodeling, we studied pigs 8–15 hr after birth. In <1 min following euthanasia, we removed and placed tracheal segments in a non-aqueous fixative containing osmium tetroxide to rapidly preserve the morphology of the airway surface (Matsui et al., 1998; Satir, 1963; Sims and Horne, 1997). The depth of periciliary liquid showed substantial variability in both non-CF and CF epithelia, with areas of deeper liquid and outstretched cilia and shallower areas with cilia that appeared bent over (Figure 3D). Therefore, we examined multiple portions of trachea, prepared multiple sections from each portion, and made many measurements from each section. Observers unaware of genotype measured periciliary liquid depth. A histogram of periciliary liquid depth is shown in Figure 3F; the mean depths of non-CF (4.5 ± 0.3 μm, n = 8 pigs) and CF (4.4 ± 0.2 μm, n = 5 pigs) periciliary liquid did not differ statistically. In addition, we prepared thin sections from the same blocks and examined them with transmission electron microscopy. The transmission electron microscopic images provided a smaller area for observation than light microscopic images and the number of samples was lower. These images also revealed both erect and bent cilia and heterogeneity in the depth of periciliary liquid covering airways of both genotypes (Figure 3E). The periciliary liquid depth was not statistically different between non-CF (4.0 ± 0.3 μm, n = 5 pigs) and CF (4.7 ± 0.3 μm, n = 5 pigs) epithelia (Figure 3G).

Compared to earlier studies, our measurements of periciliary liquid depth have the advantages that the epithelia were in vivo rather than cultured, they were immediately prepared without other manipulations, the epithelia did not demonstrate inflammation from chronic infection, and the experiments were performed at a time point when bacterial eradication was impaired. Our data also agree with an earlier study of maximal cilia length in formalin fixed/paraffin embedded newborn porcine airway epithelia, which showed no difference between CF and non-CF (Meyerholz et al., 2010a). Potential differences with a study of bronchoscopic biopsies in patients with acute and chronic disease (Griesenbach et al., 2010) raise interesting questions of whether inflammation with its associated effects on surface epithelium and submucosal glands might change ion transport or periciliary liquid height. Although our data show no difference in periciliary liquid depth between CF and non-CF newborn pigs, it is possible that with time and progression of disease, the depth of periciliary liquid might differ between the genotypes. In addition, although we measured periciliary liquid depth in trachea because of the speed with which we could remove and prepare the tissue, it will also be important to study its depth in distal airways.

All these measurements indicated that Na^+ absorption by CF tracheal/bronchial epithelia did not exceed that in non-CF. Strikingly, this was also true in nasal epithelia. So why in nasal epithelia are Vt and $\Delta Vt_{amiloride}$ increased in CF? To answer this question, we first studied cultured and excised epithelia and examined electrophysiological properties (Vt, Isc, and Gt) that are influenced by apical Na^+ conductance. Those results, considered together with an equivalent circuit model of the epithelium suggested an explanation for why electrophysiological properties differ between CF and non-CF nasal epithelia even though Na^+ absorption is not increased. We then tested predictions of that analysis.

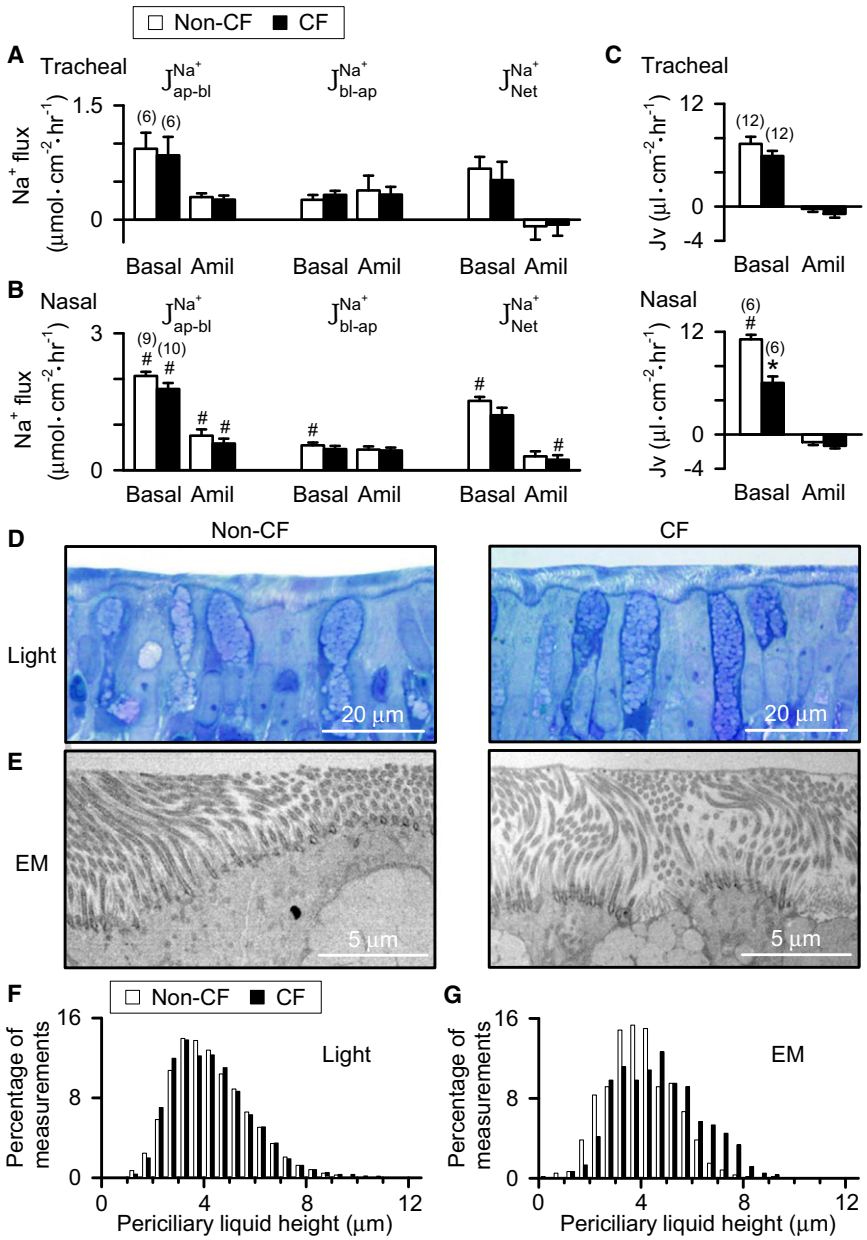

Figure 3. Porcine CF Epithelia Do Not Hyperabsorb Na^+

Data are means ± SE from newborn *CFTR*$^{+/+}$ (open bars) and *CFTR*$^{-/-}$ (closed bars) pigs. Numbers in parentheses indicate n; *p < 0.05.

(A and B) Isotopic $^{22}Na^+$ unidirectional and net Na^+ flux rates under basal conditions and after adding 100 μM amiloride apically. $J^{Na^+}_{ap-bl}$ indicates Na^+ flux from the apical (ap) to the basolateral (bl) surface, $J^{Na^+}_{bl-ap}$ indicates flux in the opposite direction, and $J^{Na^+}_{net}$ indicates net flux. # indicates that value in nasal epithelia differed from that in tracheal epithelia, p < 0.05.

(C) Rate of liquid absorption (Jv) in differentiated primary cultures of nasal and tracheal epithelia under basal conditions and after adding 100 μM amiloride apically. # indicates that value in nasal epithelia differed from that in tracheal epithelia, p < 0.05. In panels (A)–(C), the basal electrophysiological properties of matched epithelia are shown in Table S1.

(D) Examples of light microscopic images of tracheal epithelia. Note heterogeneity in depth of periciliary liquid in both non-CF and CF epithelia.

(E) Examples of transmission electron microscopic images of tracheal epithelia showing periciliary liquid.

(F) Histogram of periciliary liquid depth over tracheal epithelia obtained from light microscopic images. n = 9140 non-CF and 6260 CF measurements. Multiple images were made from each of four segments of trachea obtained from eight non-CF and 5 CF animals. See Experimental Procedures for additional details. Three observers unaware of genotype then measured periciliary liquid depth using a standardized protocol. A linear mixed model and maximum likelihood estimation were used to calculate means and standard errors allowing for variability between observers, measurements, images, segments and pigs. There was no significant difference between periciliary liquid depth in non-CF and CF epithelia (p = 0.96), and the difference was 0.71 μm or less with 95% confidence. The residual variability on the same image had an estimated standard deviation of 1.29 μm and between images was 0.60 μm. For comparison, non-CF trachea was air-exposed and showed a reduced height of periciliary liquid (2.81 μm).

(G) Histogram of periciliary liquid depth measured from transmission electron microscopic images. n = 600 measurements for each genotype and 5 animals per genotype. There was no significant difference in periciliary liquid depth between non-CF and CF, p = 0.12. For comparison the standard deviations of measurements on an image and between images were both 0.95 μm.

Vt, Isc, Gt and the Response to Amiloride under Basal Conditions in Nasal Epithelia

Transepithelial Voltage

In nasal epithelia, basal Vt was greater in CF than non-CF, both in excised and cultured epithelia (Figures 4A and 4D). $\Delta Vt_{amiloride}$ was also greater in CF than non-CF nasal epithelia. Absolute values of Vt were less in excised than in vivo and cultured epithelia, because of damage caused by clamping epithelia in Ussing chambers, i.e., "edge damage" (Helman and Miller, 1973). Excised and cultured tracheal/bronchial epithelia showed smaller or no differences between CF and non-CF (Figures S2A and S2D).

Thus, like in vivo measurements, airway location influenced whether Vt differed between CF and non-CF epithelia. This similarity suggests that cultured and excised epithelia reflect in vivo transport. However, Vt does not measure rates of ion transport.

Short-Circuit Current

In nasal epithelia, basal Isc and the amiloride-induced reduction in Isc ($\Delta Isc_{amiloride}$) were greater in CF than non-CF

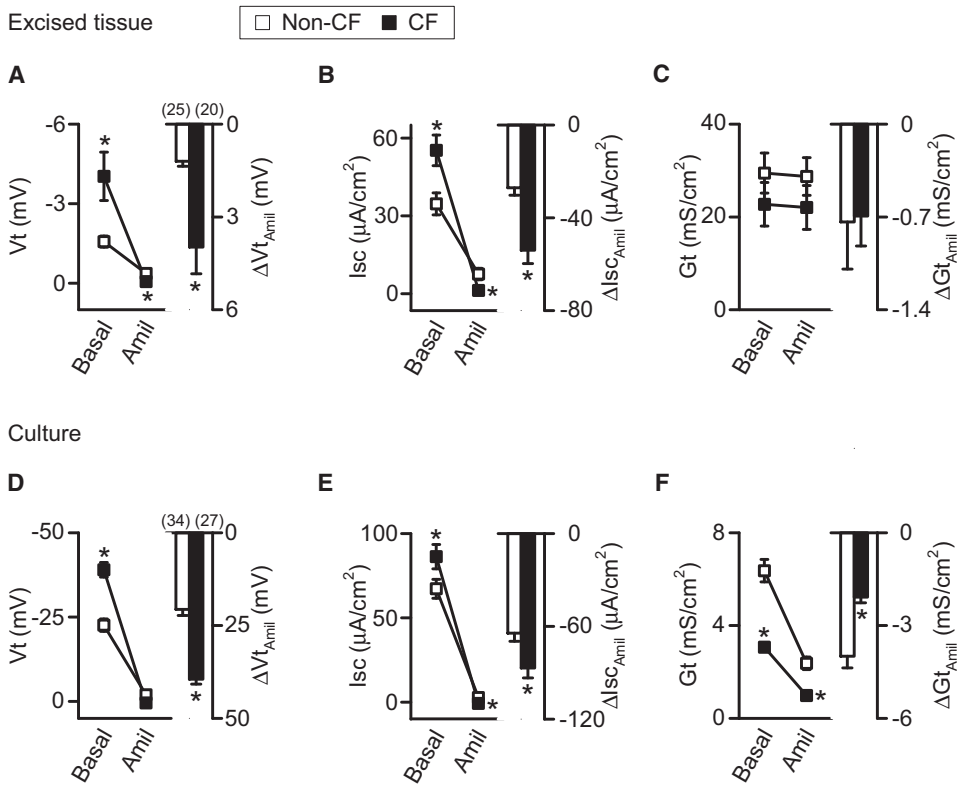

Figure 4. Amiloride Alters Electrical Properties in Non-CF and CF Nasal Epithelia

Data are means ± SE from *CFTR*$^{+/+}$ (open symbols and bars) and *CFTR*$^{-/-}$ (closed symbols and bars) pigs. Numbers in parentheses indicate n; and *p < 0.05. (A–F) Effects of adding amiloride (100 μM) to the apical solution on Vt, Isc, and Gt of freshly excised (A–C) and differentiated primary cultures (D–F) of nasal epithelia. ΔVt_{amil}, ΔIsc_{amil}, and ΔGt_{amil} indicate changes induced by amiloride. See also Figure S2.

(Figures 4B and 4E). This was the case for both excised and cultured epithelia. In tracheal/bronchial epithelia, basal Isc did not significantly differ between CF and non-CF; $\Delta Isc_{amiloride}$ was greater in excised but not cultured CF epithelia (Figures S2B and S2E).

These results largely parallel the Vt measurements, indicating a strong effect of airway region on these electrical measurements. Studies of excised human epithelia reported that CF nasal epithelia had either higher or the same Isc values as non-CF epithelia (Boucher et al., 1986; Knowles et al., 1983; Mall et al., 1998).

Transepithelial Conductance

In excised nasal epithelia, there was little difference between CF and non-CF basal Gt, perhaps because the large Gt values associated with edge damage obscured small differences (Figure 4C). However in cultured epithelia, basal Gt was greater in non-CF epithelia (Figure 4F); this can be explained by the presence of CFTR anion channels. Most importantly for assessing Na^+ permeability, the amiloride-induced decrease in Gt ($\Delta Gt_{amiloride}$) in CF did not exceed that in non-CF epithelia (Figures 4C and 4F). Likewise, $\Delta Gt_{amiloride}$ of CF tracheal/bronchial epithelia did not exceed that in non-CF (Figures S2C and S2F).

Electrical conductance is directly related to the ion permeability of channels, and $\Delta Gt_{amiloride}$ is directly influenced by the Na^+ conductance. Gt is also a more direct function of permeability than Vt or Isc, both of which are much more strongly determined by ion concentration gradients and membrane voltages. If CF epithelia had a greater apical Na^+ conductance than non-CF epithelia, and other conductances (except for the CFTR Cl^- conductance) were equal, then $\Delta Gt_{amiloride}$ should have been greater in CF. That was not the case (Figures 4C and 4F). Indeed, even if apical Na^+ conductance were equal in CF and non-CF epithelia, then $\Delta Gt_{amiloride}$ should have been greater in CF than non-CF epithelia (Extended Results, Note S1). Thus, finding that $\Delta Gt_{amiloride}$ was not greater in CF epithelia suggests that Na^+ conductance might be less in CF than non-CF epithelia.

The lack of a greater $\Delta Gt_{amiloride}$ in CF than non-CF nasal epithelia is consistent with the lack of greater Na^+ absorption measured with Na^+ fluxes and volume absorption. However, basal Vt, $\Delta Vt_{amiloride}$, basal Isc, and $\Delta Isc_{amiloride}$ were greater in CF than non-CF nasal epithelia. Because those differences are commonly interpreted to demonstrate that CF epithelia have an increased Na^+ permeability and hyperabsorb Na^+ (Boucher, 2007; Boucher et al., 1988; Donaldson and Boucher, 2007; Knowles et al., 1981), it was important to understand what causes the CF/non-CF difference in electrical properties in nasal epithelia.

Equivalent Electrical Circuit Analyses Indicate that Apical Cl^- Conductance Can Alter Vt, Isc, and the Response to Amiloride without a Change in Na^+ Permeability

Could loss of CFTR increase Vt, $\Delta Vt_{amiloride}$, Isc, and $\Delta Isc_{amiloride}$ without increasing apical Na^+ permeability? A simple explanation for how this could occur arises from the fact that placing a second ion channel (i.e., a CFTR anion conductance) in the apical membrane in parallel with a Na^+ conductance changes apical membrane voltage in three ways. First, it introduces another electromotive force generated by transmembrane ion concentration gradients. Second, it introduces a conductance that can shunt the voltage generated by other apical membrane channels, transporters, and pumps. Third, it alters the effect on apical voltage of current that is generated at the basolateral membrane. The resulting changes in apical voltage (as well as basolateral voltage) can then alter transepithelial Vt and Isc.

Horisberger (Horisberger, 2003) developed an equivalent electrical circuit model to simulate the effect of an apical membrane Cl^- conductance (CFTR) on electrical properties that are influenced by ENaC-mediated Na^+ conductance. He showed that activating CFTR reduced $\Delta Vt_{amiloride}$ and $\Delta Isc_{amiloride}$ even when Na^+ conductance was held constant. He concluded that a decrease in $\Delta Isc_{amiloride}$ or $\Delta Vt_{amiloride}$ upon CFTR activation could not be interpreted to indicate a regulatory interaction between CFTR and ENaC. Another mathematical model also showed that increasing apical Cl^- permeability could reduce Na^+ transport under short-circuited conditions even though apical Na^+ permeability remained unchanged (Duszyk and French, 1991). Thus, increasing apical Cl^- conductance can reduce Vt and Isc without a change in Na^+ conductance. Conversely, eliminating an apical Cl^- conductance, as in CF, can increase Vt, $\Delta Vt_{amiloride}$, Isc and $\Delta Isc_{amiloride}$ even without changing Na^+ conductance. Of course, in these models, changes in electrophysiological properties will depend on the absolute values of the Cl^- and Na^+ conductances and electromotive forces relative to that of all the other channels and transporters.

CFTR-Mediated Cl^- Conductance Is Greater in Nasal versus Tracheal/Bronchial Epithelia

Based on the equivalent circuit analysis, we reasoned that if nasal epithelia had a greater basal Cl^- conductance than tracheal/bronchial epithelia, it might explain the CF/non-CF difference in Vt, $\Delta Vt_{amiloride}$, Isc and $\Delta Isc_{amiloride}$ even though nasal epithelia did not hyperabsorb Na^+. To further test this possibility, we added amiloride to eliminate the Na^+ conductance and then compared Gt in non-CF and CF epithelia. The difference between non-CF and CF Gt was much greater in nasal than tracheal epithelia (Figure 5A), indicating that nasal epithelia have a greater Cl^- conductance under basal conditions. As another test, we added amiloride to inhibit Na^+ channels and DIDS to inhibit other Cl^- channels, and we then examined the response to GlyH-101 (Figure 5B). GlyH-101 reduced Gt more in nasal than tracheal epithelia, indicating that nasal epithelia have a greater CFTR Cl^- conductance under basal conditions. In addition, quantitative RT-PCR (q-RT-PCR) revealed relatively more *CFTR* transcripts in cultured nasal than tracheal epithelia (Figure 5C).

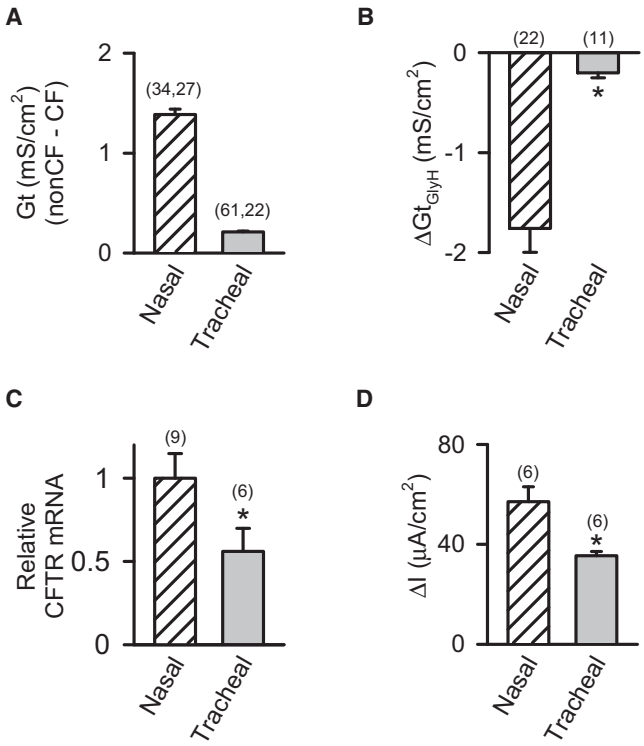

Figure 5. Non-CF Nasal Epithelia Have a Larger Cl^- Conductance Than Tracheal/Bronchial Epithelia

Data are means ± SE from nasal (cross-hatched bars) and tracheal/bronchial (shaded bars) epithelia. Amiloride (100 μM) was present on the apical surface in panels (A), (B), and (D). Numbers in parentheses indicate n; *$p < 0.05$.

(A) Difference between Gt in cultured non-CF and CF epithelia.

(B) Change in Gt (ΔGt_{GlyH}) following addition of 100 μM GlyH-101 to cultured non-CF epithelia.

(C) Relative CFTR mRNA by q-RT-PCR in primary cultures of non-CF epithelia.

(D) Apical Cl^- currents measured in nasal and tracheal epithelia from non-CF cultured epithelia. Apical solution was Cl^--free with 100 μM amiloride, 100 μM DIDS, 10 μM forskolin, and 100 μM IBMX, and basolateral solution contained 139.8 mM Cl^-. Data are current following permeabilization of basolateral membrane with nystatin (0.36 $mg.ml^{-1}$).

See also Figure S3.

The data suggested that apical Cl^- conductance under stimulated conditions was also greater in nasal than tracheal/bronchial epithelia. First, forskolin and IBMX increased Gt approximately twice as much in nasal as in tracheal/bronchial epithelia from normal pigs (Figures 1F and 1H). Second, adding GlyH-101 (after forskolin and IBMX) caused a greater Gt reduction in nasal epithelia (Figures 1J and 1L). The Gt response to cAMP-dependent stimulation and GlyH-101 inhibition showed similar trends in excised and cultured epithelia. Third, as an additional test of apical Cl^- conductance, we imposed a transepithelial Cl^- concentration gradient, added forskolin and IBMX, permeabilized the basolateral membrane with nystatin, and measured Cl^- current (Figure 5D). Cl^- current was greater in nasal than tracheal epithelia, indicating a greater Cl^- conductance. We

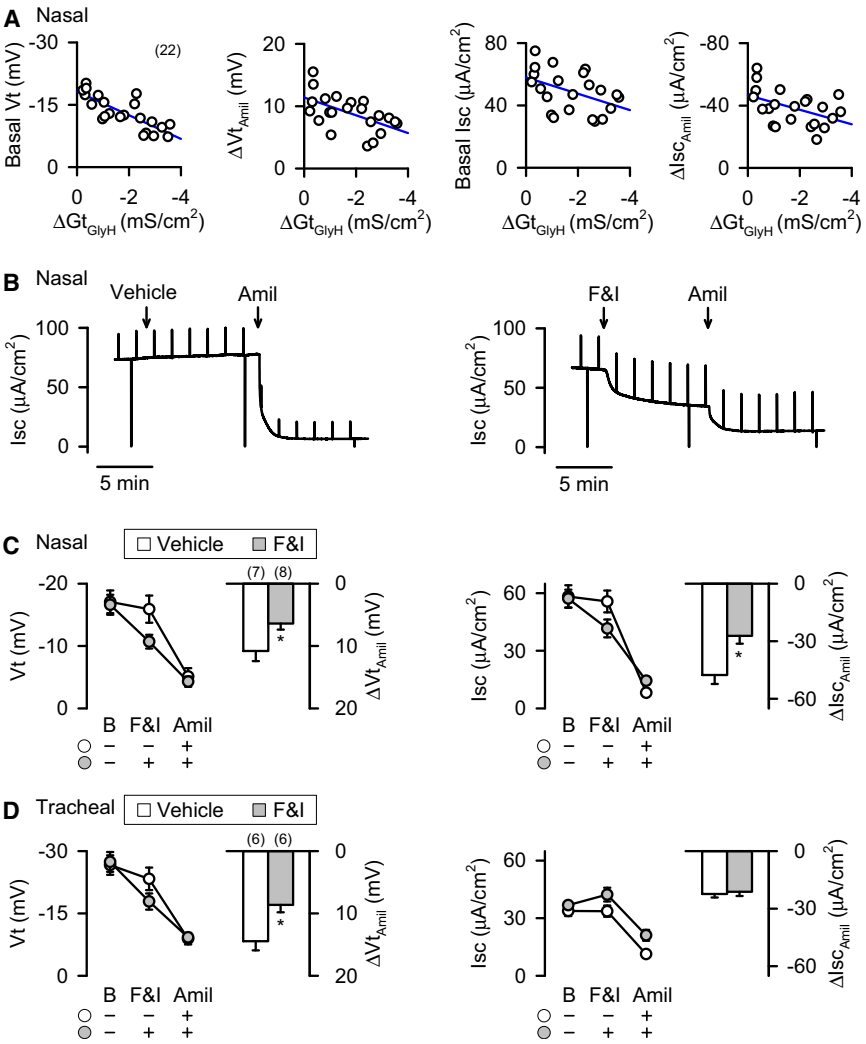

Figure 6. Increased Cl^- Conductance Is Associated with Reduced Basal and Amiloride-Sensitive Vt and Isc

(A) Relationship between basal Vt, ΔVt_{amil}, basal Isc, and ΔIsc_{amil} and the change in Gt produced by adding apical 100 μM GlyH-101 (ΔGt_{GlyH}) in the presence of amiloride. Epithelia were cultured non-CF nasal epithelia. Each data point represents a different epithelium. Blue lines indicate linear regression fits to data. Correlation coefficients and p values were: basal Vt, $R = -0.831$, $p < 0.001$; ΔVt_{amil}, $R = 0.592$, $p < 0.005$; basal Isc, $R = -0.495$, $p < 0.02$; and ΔIsc_{amil}, $R = 0.450$, $p < 0.05$. Spearman rank order correlation was used to test statistical significance.

(B and C) Effect of 10 μM forskolin and 100 μM IBMX (F&I) or vehicle control on basal Vt and Isc and on changes induced by 100 μM amiloride in cultured nasal epithelia. Panel (B) shows representative experiments, and panel (C) shows means ± SE. B, basal; *$p < 0.05$ versus vehicle controls.

(D) Same as panel (C), except tracheal epithelia.

noticed that although nasal epithelia have a greater Cl^- conductance than tracheal/bronchial epithelia, tracheal/bronchial epithelia have a greater Isc response to forskolin and IBMX when studied in the presence of amiloride; this difference appears to result from a greater driving force for Cl^- secretion that is generated by a greater basolateral K^+ conductance (Extended Results, Note S2, and Figure S3).

Thus, basal CFTR Cl^- conductance was greater in nasal than tracheal/bronchial epithelia. That result plus equivalent circuit analyses may explain why in nasal epithelia, Vt, $\Delta Vt_{amiloride}$, Isc and $\Delta Isc_{amiloride}$ are greater in CF than non-CF epithelia.

Altering Apical Cl^- Conductance Changes Vt, Isc, $\Delta Vt_{amiloride}$, and $\Delta Isc_{amiloride}$

Our conclusion that Cl^- conductance affects these electrical parameters in nasal epithelia together with the equivalent circuit analysis make four testable predictions.

First, if Vt, $\Delta Vt_{amiloride}$, Isc and $\Delta Isc_{amiloride}$ were increased in CF compared to non-CF nasal epithelia because of a lack of Cl^- conductance, then there should be an inverse relationship between these values and basal Cl^- conductance. To test this prediction, we measured Vt and Isc in nasal epithelia before and after adding amiloride. Then, to obtain an approximation of Cl^- conductance, we measured the decrease in Gt following GlyH-101 addition (ΔGt_{GlyH}). We plotted these values, which varied spontaneously from epithelium to epithelium, and found an inverse relationship (Figure 6A).

Second, further increasing apical Cl^- conductance in nasal epithelia should reduce Vt, Isc, $\Delta Vt_{amiloride}$, and $\Delta Isc_{amiloride}$. To test this prediction, we added forskolin and IBMX and found that compared to vehicle control, it decreased these electrophysiological properties in non-CF nasal epithelia (Figures 6B and 6C). The cAMP-induced reductions in Isc and Vt were opposite to the increases observed when forskolin and IBMX were added after first blocking Na^+ channels with amiloride (Figures 1C–1E and 1G). Because cAMP can increase Na^+ transport with a slow time course (Boucher et al., 1988; Cullen and Welsh, 1987), we tested this possibility by adding forskolin and IBMX to CF epithelia, where Cl^- channel activity would not confound interpretation; with this protocol, forskolin and IBMX did not alter $\Delta Isc_{amiloride}$ (-104 ± 19 μA.cm^{-2} after forskolin and IBMX versus -106 ± 11 μA.cm^{-2} with vehicle control, n = 6 each).

Although tracheal epithelia showed little difference in electrophysiological properties between CF and non-CF, we also tested the effect of forskolin and IBMX in non-CF tracheal epithelia. As in nasal epithelia, increasing cAMP reduced $\Delta Vt_{amiloride}$ (Figure 6D), due largely to an increase in Gt (change in Gt with vehicle +0.26 ± 0.04 mS.cm^{-2} and with forskolin and IBMX +2.40 ± 0.25 mS.cm^{-2}, n = 6, $p < 0.001$). However,

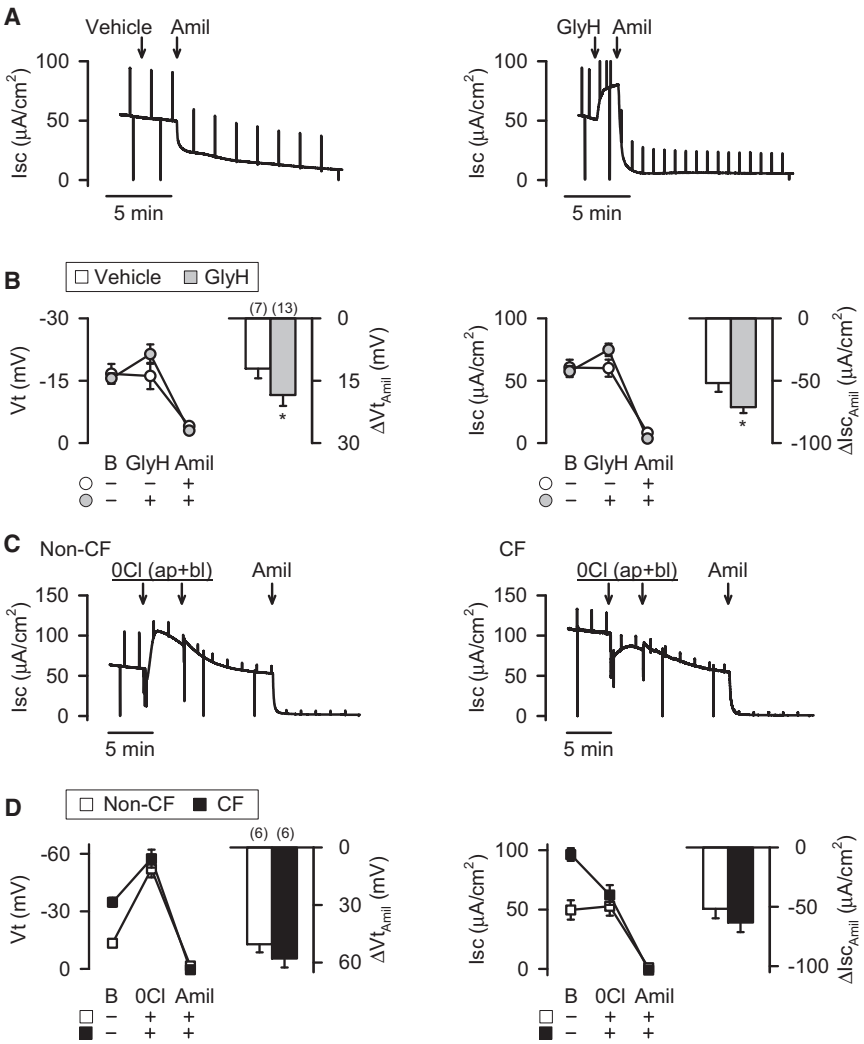

Figure 7. A Decreased Cl^- Conductance Reduces the Difference between CF and Non-CF Vt and Isc

Epithelia were cultured non-CF nasal epithelia.

(A and B) Effect of GlyH-101 (100 μM) on Vt and Isc and the response to 100 μM amiloride. Panel (A) shows representative experiments, and panel (B) shows the mean ± SE. B, basal; *$p < 0.05$ versus vehicle controls.

(C and D) Effect of Cl^--free apical (ap) and basolateral (bl) solutions on the response to amiloride in non-CF and CF epithelia. Panel (C) shows representative experiments in non-CF (left) and CF (right) epithelia, and panel (D) shows means ± SE. The two arrows for the change to Cl^--free solution in panel (C) indicate two exchanges of bathing solution.

$\Delta Isc_{amiloride}$ did not change, perhaps because tracheal epithelia may have greater membrane driving forces for Cl^- secretion under short-circuit conditions (Extended Results, Note S2). These results further indicate that the electrophysiological properties are affected by factors other than just Na^+ permeability. These results may also explain two earlier studies that reported that increasing cAMP increased Isc or calculated current in human nasal epithelia (Boucher et al., 1988, 1986).

Third, decreasing apical Cl^- conductance in nasal epithelia should increase Vt, Isc, $\Delta Vt_{amiloride}$, and $\Delta Isc_{amiloride}$. Adding GlyH-101 to reduce the Cl^- conductance of non-CF epithelia acutely increased these properties (Figures 7A and 7B). Note that the increase in Isc and Vt are opposite to what occurs when we added GlyH-101 in the presence of amiloride, which eliminates the Na^+ conductance (Figures 1I and 1K).

Fourth, non-CF and CF epithelia should show similar properties when Cl^- conductance is eliminated by replacing Cl^- with gluconate, an impermeant anion. In CF nasal epithelia, Vt and Isc were approximately double the values of non-CF epithelia (Figures 7C and 7D). However, in a Cl^-- and HCO_3^--free solution, those values and $\Delta Vt_{amiloride}$ and $\Delta Isc_{amiloride}$ did not differ between genotypes.

These data further clarify how electrophysiological measurements (increased Vt, $\Delta Vt_{amiloride}$, Isc and $\Delta Isc_{amiloride}$) that are often interpreted to demonstrate increased CF Na^+ absorption may simply reflect the lack of a Cl^- conductance.

DISCUSSION

Advantages, Limitations, and Considerations of This Study

Our work has the advantage that we studied airway epithelia in vivo, in freshly excised tissue, and in primary cultures of differentiated airway epithelia, and we obtained similar results. In this regard, most studies of CF ion transport have relied either on in vivo nasal Vt or on cultured airway epithelia or cell lines. However, the relationship between the quantitative and qualitative aspects of ion transport in vivo and those measured in cultured airway epithelia have been uncertain. In addition, chronic infection and inflammation may influence measures of ion transport in nasal Vt, in excised tissue, and perhaps in epithelia cultured from patients (Fu et al., 2007; Gray et al., 2004; Kunzelmann et al., 2006). Thus, it is encouraging that our data from newborn pigs indicate that primary airway cultures retain many of the properties of in vivo and excised airways. For example, like excised nasal epithelia, cultured epithelia derived from nasal tissue had a greater CFTR Cl^- conductance than tracheal/bronchial epithelia. In addition, the response to interventions was also similar in vivo, in excised tissue, and in differentiated cultures. These data suggest that cultured epithelia provide a valuable model for studying electrolyte transport by porcine airway.

In this study, we primarily investigated CFTR-mediated anion conductance and amiloride-sensitive Na^+ conductance.

However, numerous other channels and transporters may contribute to electrolyte transport across airway epithelia, including SLC26 transporters, other HCO_3^- transporters, electrically neutral Na^+ transporters, K^+ channels and the Na^+/K^+-ATPase. Ca^{2+}-activated Cl^- channels are also of interest because it has been speculated that they might compensate for the loss of CFTR anion channels in *CFTR*$^{-/-}$ mice, thereby accounting for lack of a typical CF phenotype (Clarke et al., 1994). Although our data do not indicate whether or not these other transport processes are altered by loss of CFTR, their function remains an important area for investigation.

Our conclusions also have limitations. In comparing how loss of CFTR function affects Na^+ absorption in pigs and humans, we acknowledge that regulation of Na^+ absorption might differ between the two species, i.e., human CF airway epithelia might hyperabsorb Na^+, whereas porcine airway epithelia do not. In addition, although we studied newborn pigs that exhibit a bacterial host defense defect, it is possible that epithelial transport properties differ in older animals and adults. We also studied *CFTR*$^{-/-}$ pigs, whereas most patients have at least one *ΔF508* allele (Welsh et al., 2001). We previously showed that human, porcine, and murine CFTR-ΔF508 show some differences in processing (Ostedgaard et al., 2007), and thus, it should be interesting to learn how *CFTR*$^{\Delta F508/\Delta F508}$ pigs compare to *CFTR*$^{-/-}$ pigs.

There are additional considerations from our studies. First, although we found that CF epithelia do not hyperabsorb Na^+, in vivo measures of basal Vt and $\Delta Vt_{amiloride}$ can be valuable assays in the diagnosis of CF and for assessing the response to interventions designed to increase CFTR activity in patients with CF (Standaert et al., 2004). Second, our conclusions do not mean that increased Na^+ absorption could not occur at a later time-point as disease progresses or under some conditions (Myerburg et al., 2006). Third, improving airway surface liquid hydration may benefit patients with CF (Elkins et al., 2006; Donaldson et al., 2006; Robinson et al., 1997); our study did not address that issue. Fourth, it may seem paradoxical that CF nasal epithelia have a greater $\Delta Vt_{amiloride}$ and $\Delta Isc_{amiloride}$ than non-CF epithelia, and yet Na^+ absorption is not increased in CF. As one example, consider that in non-CF epithelia studied under short-circuit conditions, adding amiloride will hyperpolarize apical membrane voltage, thereby increasing the driving force for Cl^- secretion, whereas lack of CFTR precludes Cl^- secretion in CF epithelia. Thus, adding amiloride under short-circuit conditions will inhibit Na^+ absorption and increase Cl^- secretion in non-CF epithelia, and therefore $\Delta Isc_{amiloride}$ will be greater in CF than non-CF epithelia when apical Na^+ conductance is the same. While this is not the only factor involved in determining the response to amiloride (see above), it provides an example of the complexity of interpreting electrical properties in assessing epithelial ion transport.

Implications for CF Pathogenesis and Treatments

Our data indicate that Na^+ absorption is not increased in airway epithelia from newborn CF compared to non-CF pigs. We also explain how loss of CFTR can alter electrophysiological properties that have been construed to indicate enhanced Na^+ absorption in CF. These results conflict with the widely held view that CFTR negatively regulates ENaC, and that the loss of this regulation in CF causes airway epithelia to hyperabsorb Na^+ (Boucher, 2007; Donaldson and Boucher, 2007). Although we studied electrolyte transport by airway epithelia of pigs shortly after birth, data from that time-point is germane to the issue because newborn CF pigs have an impaired ability to eliminate bacteria (Stoltz et al., 2010). Nevertheless, assaying these properties in older animals as the disease progresses will also be important.

Elucidating the first steps leading to CF lung disease is key if we are to understand pathogenesis and develop mechanism-based treatments and preventions. CF pigs provide a unique opportunity to investigate those initiating steps, because they spontaneously develop lung disease like humans, and at birth they already manifest a bacterial host defense defect, but they do not have the secondary consequences of infection. Our studies using this model identify loss of CFTR anion permeability as the predominant transport defect at birth. In this regard, porcine CF airway epithelia are similar to two other tissues that express both CFTR and ENaC channels, sweat gland ducts and submucosal glands, where loss of anion transport and not Na^+ hyperabsorption is the CF defect (Joo et al., 2006; Quinton, 1999, 2007). Thus, our data emphasize the role that loss of Cl^- and HCO_3^- permeability may play in impairing bacterial eradication and the subsequent development of airway disease.

EXPERIMENTAL PROCEDURES

For a detailed description of all the methods, please see the Extended Experimental Procedures.

CFTR$^{-/-}$ and *CFTR*$^{+/+}$ Pigs

We previously reported generation of *CFTR*$^{-/-}$ pigs (Rogers et al., 2008a, 2008b; Stoltz et al., 2010). The University of Iowa Animal Care and Use Committee approved the animal studies. Animals were produced by mating *CFTR*$^{+/-}$ male and female pigs. Newborn littermates were obtained from Exemplar Genetics. Animals were studied and/or euthanized 8–15 hr after birth (Euthasol, Virbac).

Measurement of Transepithelial Voltage In Vivo

Transepithelial voltage (Vt) was measured in the nose and trachea of newborn pigs using a standard protocol as described previously (Rogers et al., 2008b; Standaert et al., 2004).

Preparation of Differentiated Primary Cultures of Airway Epithelia

Epithelial cells were isolated from the various tissues by enzymatic digestion, seeded onto permeable filter supports, and grown at the air-liquid interface as previously described (Karp et al., 2002). Differentiated epithelia were used at least 14 days after seeding.

Electrophysiological Measurements in Freshly Excised and Cultured Epithelia

Epithelial tissues were excised from the nasal turbinate and septum, and from trachea through 2nd-generation bronchi immediately after animals were euthanized. Tissues and cultured epithelia were studied in modified Ussing chambers. Epithelia were bathed on both surfaces with solution containing (mM): 135 NaCl, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 dextrose, 5 HEPES (pH = 7.4) at 37°C and gassed with compressed air. For Cl^--free solution, Cl^- was replaced with gluconate and Ca^{2+} was increased to 5 mM. For the high K^+ and Na^+-free solution, Na^+ was replaced with K^+. To study HCO_3^- transport, we used Cl^--free Kreb's solution containing (mM): 118.9 Na-Gluconate, 25 $NaHCO_3$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 5 CaGluconate, 1 MgGluconate, and 5 dextrose and gassed with 5% CO_2.

Vt was maintained at 0 mV to measure short-circuit current (Isc). Transepithelial electrical conductance (Gt) was measured by intermittently clamping Vt to +5 and/or −5 mV. Spontaneous values of Vt were measured by transiently removing the voltage clamp. At the beginning of these experiments, we used cultured, non-CF tracheal epithelia to test the dose-response relationship for the agents used in this study (Figure S1).

Measurement of Na^+ Flux and Fluid Transport

Transepithelial Na^+ flux and liquid absorption were measured using methods similar to those we previously reported (Flynn et al., 2009; Zabner et al., 1998). The supplemental methods describe the detailed methods.

Measurement of Periciliary Liquid Depth

Newborn pigs (8–15 hr old) were sedated with ketamine and xylazine (15–20 mg/kg and 1.5 mg/kg, IM, respectively) and immediately euthanized with intravenous Euthasol. A 1–2 cm portion of the trachea was immediately removed, immersed in 2% osmium tetroxide dissolved in FC-72 perfluorocarbon (3M, St Paul, MN), and fixed for 90–120 min. The trachea was then rinsed in FC-72 and dehydrated in three changes of 100% ethanol, one hr each. During the second ethanol step, the samples were hand-trimmed into four pieces with a scalpel to 1 mm slices. Both open ends of the tracheas were removed and discarded to avoid areas possibly disturbed during removal from the animal. Tissue near the trachealis muscle was avoided. After dehydration, samples were placed in 2:1 100% ethanol:Eponate 12 resin (Ted Pella, Inc., Redding, CA) followed by 1:2 100% ethanol:Eponate 12 for one hr each. Tracheal segments were then infiltrated in 3 changes of 100% Eponate 12 for at least 2 hr each and polymerized for 24 hr at 60°C.

Following processing, four tissue blocks from each trachea were trimmed and thick-sectioned for light-level PCL thickness determination after staining with Toluidine Blue. Imaging was performed on an Olympus BX-51 equipped with a DP-72 CCD camera (Olympus America Inc., Center Valley, PA) using a 100× NA 1.35 PlanApo lens. Five random images were taken from each block and PCL measured using ImageJ (NIH, Bethesda, MD). PCL height was determined by drawing a line perpendicular to the apical membrane of the epithelial cell surface. On each image, PCL height measurements were performed at 20 random locations. Three observers who were unaware of the *CFTR* genotype made independent measurements on every image (number of approximate measurements per trachea: four tracheal blocks/animal × 5 images/block × 20 measurements/image ~400 PCL measurements/piglet trachea/observer). Measurements were made by three independent observers; therefore ~1200 PCL measurements/piglet trachea were obtained. A linear mixed model and maximum likelihood estimation were used to estimate means and standard errors (Bates and Maechler, 2009; R Development Core Team, 2009). The model included fixed effects for observers and genotype and random effects for pigs, segments of the trachea within a pig, images within a segment, and inter-observer variability of measurements on the same image. Tissue blocks used for light microscopy were also trimmed and sectioned at 80 nm for transmission electron microscopy. Non poststained grids were imaged in a JEOL 1230 TEM (JEOL USA Inc., Peabody, MA) equipped with a Gatan 2k × 2k camera (Gatan Inc., Pleasanton, CA). The transmission electron microscope data were analyzed similarly to the light microscopic images.

Quantitative Real-Time RT-PCR

Total RNA from excised tissue and cultures was isolated and prepared using standard techniques. Table S2 shows the PCR primers. Real-time RT-PCR was performed using standard methodology and analysis.

Statistical Analysis

Data are presented as means ± standard error (SE). Spearman rank order correlation was used to test statistical significance of relationships shown in Figure 6A. The methods for statistical evaluation of periciliary liquid depth are described in that section of the methods. All other statistical analysis used an unpaired t test. Differences were considered statistically significant at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Results, Extended Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2010.11.029.

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