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Liquid Movement Across the Surface Epithelium of Large Airways

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Abstract

The cystic fibrosis conductance regulator *CFTR* gene is found on chromosome 7 (Kerem et al., 1989; Riordan et al., 1989) and encodes for a 1,480 amino acid protein which is present in the plasma membrane of epithelial cells (Anderson et al., 1992). This protein appears to have many functions, but a unifying theme is that it acts as a protein kinase C- and cyclic AMP-regulated Cl^- channel (Winpenny et al., 1995; Jia et al., 1997). In the superficial epithelium of the conducting airways, CFTR is involved in Cl^- secretion (Boucher, 2003) and also acts as a regulator of the epithelial Na^+ channel (ENaC) and hence Na^+ absorption (Boucher et al., 1986; Stutts et al., 1995). In this chapter, we will discuss the regulation of these two ion channels, and how they can influence liquid movement across the superficial airway epithelium.

Cystic Fibrosis Lung Disease

Elucidation of ion transport in large airways has been advanced substantially by studying the pathophysiological effects of cystic fibrosis (CF) in the airways (a disease caused by an ion transport defect in the epithelium), so it is useful to consider this condition in the first instance when discussing liquid movement across the surface epithelium of large airways.

CF reflects a spectrum of more than 1,400 mutations in the cystic fibrosis conductance regulator (CFTR) gene (Davis, 2006). The most common mutation, which occurs on 70% of all CF chromosomes, is deletion of phenylalanine at position 508 of the CFTR protein, which is designated as ΔF508. The ΔF508 CFTR protein falls foul of the processing pathways and is degraded by a variety of intracellular pathways. The molecular pathogenesis of this most common form of CFTR mutation reflects the absence of functioning CFTR proteins in the apical plasma membrane of airway epithelia. Other mutations can produce a non-functioning CFTR Cl^- channel at the apical membrane and/or a mutant CFTR with abnormal ion permeation characteristics.

CF lung disease reflects a failure of airway epithelia to adequately hydrate mucus on their surfaces, particularly in response to infectious or toxic challenges. This lack of mucus hydration leads to reduced mucus clearance, adhesion of mucus to airway surfaces, and chronic bacterial infection of the lung (Knowles and Boucher, 2002; Chmiel and Davis, 2003). The concept that airway surface dehydration can produce CF-like lung disease was tested in an *in vivo* mouse model by transgenically overexpressing subunits of the epithelial Na^+ channel (ENaC) in an attempt to shift the balance of salt and water movement away from Cl^- secretion and towards Na^+ absorption (Mall et al., 2004). Further investigation of these mice overexpressing the

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βENaC subunit demonstrated that Na^+ absorption was increased three-fold over controls, whereas the ability to generate Cl^- secretion was unaltered. The disequilibrium between absorption and secretion produced the predicted depletion of airway surface liquid (ASL) volume, which induced mucus stasis, and death due to mucus obstruction in ~50% of transgenic animals. Interestingly, the reduction in ASL volume produced the neutrophilic inflammation and goblet cell hyperplasia which are characteristic of CF lung disease. Thus, these observations provided strong evidence that a disturbance of the balance between Na^+ absorption and Cl^- secretion could produce airway surface dehydration and induce the inflammation that is typical of CF lung disease.

Additionally, the submucosal glands are a potential source of airway surface liquid/mucus in the proximal airways (Ballard and Inglis, 2004; Wine and Joo, 2004). In normal airways CFTR mediates anion secretion in submucosal glands to drive liquid secretion. When secretions are inhibited using Cl^- and HCO_3^- inhibitors *in vitro*, the resultant changes appear similar to those observed in early CF airway disease, i.e. dehydrated ASL and mucus plugging in glands (Ballard et al., 2002; Trout et al., 2003). Opinion is divided regarding whether ASL dehydration in CF airway disease is due to altered epithelial ion transport or disturbance of submucosal gland secretion with evidence linking both phenomena to the manifestation of the disease (Chmiel and Davis, 2003; Wine and Joo, 2004; Inglis and Wilson, 2005; Boucher, 2007). However, due to a lack of specific reagents to differentiate between glandular and superficial secretions, it has been hard to delineate the relative contributions of the two sources to airway hydration.

Interestingly, submucosal glands are virtually absent in the distal airways (Jeffery, 1983). However, some of the earliest lesions in CF airways (i.e. mucus plugs) occur in these glandless small airways (Moskowitz et al., 2005), suggesting that altered ion transport in the superficial epithelia has important consequences in lung defense and the progression of CF lung disease.

Regulation of ASL hydration under static culture conditions

The hydration of the normal airway surface, i.e., the volume of ASL, is maintained in the highly water-permeable airway epithelia by active ion transport processes that control the mass of salt (NaCl) on airway surfaces, with water following passively by osmosis (Matsui et al., 2000). Under conditions where airway surfaces are bathed with large volumes of liquid, which may experimentally follow the addition of Ringer solution airways mounted in Ussing chambers, normal airway epithelia have the capacity to remove (absorb) this excess liquid. The absorptive pathway reflects the capacity to actively absorb Na^+ ions, with Na^+ entering the cell from ASL through ENaC. Na^+ ions exit the cell via the basolateral Na^+-K^+-ATPase with Cl^- following passively through the tight junctions. Chloride may be secreted from the cell by an apical membrane CFTR Cl^- channel, with Cl^- entering the cell principally via a Na^+-K^+-2Cl- cotransporter. Regulation of the balance between absorption and secretion determines the net transport of ions across the epithelium and, hence, the mass of salt on an epithelial surface.

Data from an *in vitro* well-differentiated cell culture model interfaced to a confocal microscope to measure ASL volume have been key in elucidating the importance of airway surface hydration in lung defense (Matsui et al., 1998). This technique has the advantage of being able to study live airway epithelial cultures with intact native ASL under thin film conditions (i.e. ~7 μm in height) and builds on the existing knowledge of native ASL which has previously been garnered by fixing airways *in situ* to image ASL by conventional light and electron microscopy techniques (Sims and Horne, 1997; Wu et al., 1998). This technique has been previously described (i.e. Tarran and Boucher, 2002) and will only briefly be reviewed here: ASL is pre-labeled with fluorescent dextrans (e.g. 10 kD Dextran conjugated to Texas Red) which are trapped in the ASL compartment. A bolus of solution (e.g. 20 μl PBS) containing

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the fluorescent dextran can be added and how the epithelia can modify its volume/composition can be observed with time. Also, ion channel modulators (e.g. amiloride) can be added to this solution at this time. Alternatively, once the test solution has been added to the ASL, excess solution can be aspirated with a Pasteur pipette to yield a lower starting volume. We estimate that the ASL volume of an airway epithelial culture grown on a 12 mm insert is ≤ 10 μl. Thus, the washing/addition of the test solution can be used to replace the ASL with a test solution of known composition. Since ASL can easily evaporate, perfluorocarbon (PFC) is typically placed over the ASL whilst live imaging occurs. PFC is highly oxygen-permeable and does not directly affect ASL height, mucus or ion transport rates (Tarran and Boucher, 2002).

We have previously shown that normal airway epithelia were capable of absorbing "excess" ASL to bring ASL volume down to a height that approximates the height of outstretched cilia, i.e. 7 μm, whilst CF epithelia were unable to regulate ASL volume and ASL height collapsed to ~3 μm, a height incompatible with efficient mucus transport (Fig. 1 A,B; (Matsui et al., 1998; Tarran et al., 2005). This event was accompanied by a shift from Na^+ absorption to Cl^- secretion by normal airway epithelia that was absent from CF airways (Tarran et al., 2005). Inhibition of Cl^- secretion by bumetanide caused a collapse of normal ASL height in a similar fashion to CF airways, suggesting that the increase in Cl^- secretion was a necessary adjustment to diminished ASL height (Tarran et al., 2006b). Similarly, a decline in Na^+ absorption was also required to maintain ASL height, as demonstrated by the addition of a cationophore (nystatin), that allows unregulated Na^+ influx, resulting in a collapse in normal ASL height (Fig. 1C; (Tarran et al., 2006b). Neither of these manoeuvres had any effect on CF airway cultures, reflecting the lack of Cl^- secretion and a failure to regulate Na^+ absorption.

In comparison to Cl^- (~120 mM; (Tarran et al., 2001b), HCO_3^- is present in the ASL in relatively low quantities (< 10 mM) (Coakley et al., 2003) and is not therefore present in sufficient quantities to alter ASL height directly, although HCO_3^- secretion is predicted to have large effects on ASL pH, which may alter ion channel and ecto-enzyme function and indirectly alter ASL height. However, these experiments have yet to be performed and the remainder of this chapter will focus primarily on Cl^- transport which has been more extensively studied and has direct effects on ASL height.

Regulation of Cl^- secretion by nucleotides and nucleosides

ATP, interacting with mucosal $P2Y_2$ receptors ($P2Y_2$-R) can inhibit the rate of Na^+ absorption and initiate/accelerate Cl^- secretion via PKC-mediated actions on CFTR and Ca^{2+}-mediated actions on calcium-activated chloride channels (CaCC). It has been demonstrated that mucosal nucleotide addition to airway epithelia induces ASL secretion in both normal and CF airways (Tarran et al., 2001b; Tarran et al., 2002). However, the effects are relatively short-lived and ASL height returns to baseline values within 1 h, consistent with Ussing chamber measurements of Ca^{2+}-mediated anion-secretion (Clarke et al., 1994; Grubb et al., 1994). This short action may be explained by the rapid degradation (~30s) (Tarran et al., 2001b) of even very large doses of ATP by ATPases that are active within the ASL (Picher and Boucher, 2003) and may also be due to desensitization of the $P2Y_2$-R or transience of the IP_3/Ca^{2+} signal (Lazarowski and Boucher, 2001). CFTR may be stimulated in an autocrine/paracrine fashion by adenosine formed from the metabolism of ATP, ADP and AMP by ecto-nucleotidases and ecto-apyrases located in the apical membrane of the superficial epithelia (Lazarowski et al., 2004; Picher et al., 2004). Recent data have suggested that both systems are operative and important in regulating the balance of Na^+ absorption vs. Cl^- secretion in normal airway epithelia (Tarran et al., 2005).

The quantities of ATP, and its metabolic product adenosine, on airway surfaces are determined by the mechanical stresses imparted to airway epithelia during breathing that regulate the rate

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of ATP release from epithelial cells into ASL (Tarran et al., 2006a). The possession of more than one pathway to secrete Cl^- into the ASL ensures multiple redundancies and is likely to be important to ensure adequate mucus hydration under a wide range of physiological and pathophysiological conditions.

CF airway epithelia are vulnerable to dehydration because of the absence of the CFTR protein in the plasma membrane. Specifically, the adenosine-A_{2b} receptor system and cAMP-dependent activation of PKA are functional, but the absence of CFTR protein in the membrane renders the Cl^- secretory and Na^+ inhibitory effects of adenosine signaling ineffective. In contrast, the ATP-P2Y-R signaling system is effective in CF airway epithelia in inhibiting ENaC and initiating CaCC-mediated Cl^- secretion (Mall et al., 2000). *In vitro* studies demonstrate that under conditions that reprise tidal breathing *in vivo*, CF airway epithelia are covered by sufficient ASL to effect normal mucociliary clearance, but they are missing the liquid reserve found in normal airways (Tarran et al., 2005). Importantly, the ability of CF airways to hydrate mucus diminishes if the lung is confronted with circumstances that impair the efficacy of the ATP signaling system. For example, infection of CF airway epithelia with paramyxoviruses, e.g., RSV, induces CF airway epithelia to upregulate extracellular ATPase expression (Tarran et al., 2005). This increased ATPase activity is sufficient to degrade the ATP in ASL and diminish/abolish $P2Y_2$ inhibition of Na^+ absorption and stimulation of CaCC-mediated Cl^- secretion. Under these conditions, unregulated salt and liquid absorption occurs, the CF airway surface becomes dehydrated, mucus transport is abolished, and mucus stasis ensues. It has been speculated that a similar event may occur in CF patients *in vivo* during viral infections, leading to accumulation of dehydrated mucus in virus-infected areas of the lung and spread of bacterial infection from bronchiectatic areas to produce an acute exacerbation (Gilligan, 1991; Wat and Doull, 2003).

Regulation of ENaC by CFTR & cAMP

In normal human airways ENaC is inactivated by agonists which raise cAMP such as forskolin or isoproterenol (Knowles et al., 1983a; Knowles et al., 1983b; Boucher et al., 1986). These agonists also activate CFTR and induce Cl^- secretion so this inhibition of ENaC may be a mechanism to enhance Cl^- secretion. This facilitation is required since the ASL Cl^- concentration is 3-4 fold higher than intracellular Cl^- (Willumsen et al., 1989b; Tarran et al., 2001a). This unfavourable gradient can be overcome by inhibiting ENaC which hyperpolarizes the apical membrane to provide the necessary electrical driving force for cellular Cl^- exit into the ASL in an analogous fashion to the addition of amiloride *in vitro* (Boucher, 1994).

In contrast, cAMP positively regulates ENaC in CF airways with disastrous consequences: ENaC is active under basal conditions in CF airways, and following cAMP stimulation, ENaC activity is further increased resulting in Na^+ hyperabsorption (Knowles et al., 1983a; Knowles et al., 1983b; Boucher et al., 1986) and ASL depletion (Matsui et al., 1998; Tarran et al., 2005) which lead to a failure of mucus clearance.

Thus, CFTR appears to act as a “brake” by limiting ENaC activity in normal airways. However, whilst the field is replete with data supporting this hypothesis in native human tissues both *in vivo* and *ex vivo*, reports using cell culture systems have varied, suggesting that the interaction between these two ion channels is variable and not necessarily hard-wired. For example, in the sweat glands, where Cl^- absorption rather than Cl^- secretion occurs under basal conditions, ENaC is activated in parallel with CFTR rather than being inactivated by it (Reddy et al., 1999).

In a landmark study, (Stutts et al., 1995) used a mammalian heterologous expression system to show by patch-clamp that CFTR-expression did indeed alter ENaC activity following forskolin-induced increases in cAMP. Surprisingly, more than ten years later the molecular

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basis underlying this phenomenon remains obscure (Huang et al., 2004). Using the *Xenopus* oocyte expression system, Kunzelmann et al. (1997) and Ji et al. 2000) demonstrated that the intracellular domains of CFTR directly interacted with ENaC, and that these domains were sufficient to alter ENaC currents when the appropriate cRNAs were co-injected into oocytes. However, their may also be indirect interactions between these two ion channels. CFTR and ENaC are linked to the cytoskeleton (Berdiev et al., 2001; Okiyoneda and Lukacs, 2007) and CFTR-ENaC interactions have been shown to be actin-dependent (Ismailov et al., 1997). Kunzelmann et al. (1997) and Ji et al. (2000) also reported that a reduction in the intracellular Cl^- concentration following stimulation of either CFTR or ClC Cl^- channels lowers ENaC activity (Mo and Wills, 2004; Bachhuber et al., 2005) and accordingly they have proposed a model whereby ENaC is regulated by the intracellular Cl^- concentration. Despite a precedence for this type of regulation of ENaC in mouse salivary duct epithelia (Cook et al., 1998), this theory is controversial with regard to the airways and other investigators have reported that ENaC is still regulated appropriately following co-injection of αβγENaC and a non-Cl^--conducting CFTR mutant (Suaud et al., 2007). Certainly, if ENaC is regulated by intracellular Cl^-, then this raises the question of why does ENaC also need to bind to an intracellular domain of CFTR to be regulated by intracellular Cl^-.

Whilst CFTR has been thought to alter the ENaC channel open probability (P_o), other means of ENaC regulation by CFTR have recently been proposed. βENaC surface expression (i.e. the number of ENaC channels in the plasma membrane, *N*) was found to be unaffected by CFTR expression in oocytes by surface biotinylation/Western blotting (Suaud et al., 2007). However, upon stimulation of CFTR, βENaC surface expression was markedly reduced within ~20 min. Contrastingly, CFTR expression was also shown to stabilize βENaC at the plasma membrane in polarized MDCK cells (Lu et al., 2007) although these authors did not look at the effects of CFTR activation on ENaC expression levels.

Differences likely exist between oocytes, immortalized mammalian cells and polarized epithelia with regard to cAMP signaling and possible CFTR-ENaC interactions that could alter the measured outcomes. For example, whilst forskolin stimulates adenylate cyclase to increase global cAMP, Huang et al demonstrated that ADO can activate CFTR to similar levels as forskolin despite inducing much smaller changes in global cAMP (Huang et al., 2001). Phosphodiesterase 4D has been shown to form a cAMP diffusion barrier at the apical membrane of airway epithelia, and is likely a mechanism to induce very localized cAMP signaling that may be absent from other cell types. ADO, has also been shown to stimulate ENaC in the absence of CFTR (Chambers et al., 2006) and to increase ASL absorption in CF bronchial epithelial cultures (Tarran et al., 2006b). ADO has also been shown to promote arachidonic acid release from Calu-3 cells via phospholipase A2 (Cobb et al., 2001) which is also required for CFTR activation and may also vary in a cell-type specific fashion.

Many of the previous studies have used non-polarized cells to study the mechanism of CFTR-mediated inhibition of ENaC due to the difficulty of performing expression and biochemical experiments in native tissues which may have led to varying results. However, with the advent of better CFTR/ENaC antibodies and the ability to introduce shRNAs to epithelia via viral expression systems (Tong et al) it may be possible to study this issue in polarized epithelia and better understand this important interaction.

Regulation of ENaC by Channel-Activating Proteases

cAMP and CFTR are not the only regulators of ENaC, and ENaC is independently regulated by proteolytic cleavage by serine proteases. Rossier and colleagues initially identified a membrane-bound serine protease that acts as a channel activating protease which they termed CAP1 (Vallet et al., 1997; Chraibi et al., 1998; Rossier, 2004). The activation of ENaC by

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CAP1 can be mimicked by external addition of trypsin, showing that both CAP1 and trypsin act via the same pathway. Aprotinin, an inhibitor of serine proteases, can block this effect (Vallet et al., 1997; Vuagniaux et al., 2000). We have recently identified several candidate CAPs and CAP-inhibitors that are expressed in both normal and CF bronchial cultures using quantitative real time PCR and some of these proteins may be spontaneously active (Tarran et al., 2006b). For example, diluting native ASL with 20 μl Ringer solution increased the amiloride-sensitive transepithelial potential difference (V_t) and decreased trypsin-sensitivity in normal airways (Tarran et al., 2006b). However, V_t declined 24-48 h after Ringer addition and became much more sensitive to trypsin (Fig. 2A). These findings suggested (i) that CAPs are tethered to the apical membrane, close to ENaC, and are not diluted following Ringer addition and (ii) that CAP-inhibitors are soluble and can be washed away following Ringer addition. *In vivo*, an additional source of CAP-inhibitors may be the submucosal gland secretions, which when secreted onto the superficial epithelium may also regulate Na^+ absorption and hence, ASL volume (Joo et al., 2004). The aprotinin-sensitivity of normal cultures waned over 48 h, suggesting that soluble CAP-inhibitors accumulate in the ASL with time under thin film conditions (e.g. Fig. 2). Dilution of these endogenous CAP-inhibitors when large volumes of Ringer are added to the apical surface likely explains why previous investigators failed to detect trypsin sensitivity in airway epithelia mounted in Ussing chambers, as under these conditions, the volume of liquid bathing the apical surface (5 ml) likely diluted any naturally-formed inhibitors (Bridges et al., 2001; Donaldson et al., 2002).

CF airway cultures are trypsin-insensitive over the entire 48 h period shown in Fig. 2A (Tarran et al., 2006b), suggesting that ENaC was maximally activated in these cultures. The observation that V_t in CF cultures was inhibited by aprotinin suggests that ENaC responds normally to CAP-inhibitors and implies that CF cultures exhibit a failure to inhibit CAPs over time, perhaps due to a failure to accumulate functional CAP-inhibitors within the ASL (Fig. 2B). In support of this hypothesis, Myerburg *et al*., have demonstrated that the alpha subunit of ENaC undergoes increased proteolytic processing in CF airways making ENaC constitutively active, indicating that ASL volume does indeed modulate ENaC activity by modification of the serine protease-protease inhibitor balance (Myerburg et al., 2006). We do not know which CAPs or their inhibitors are active in our culture system. However, Tong et al. (Tong et al., 2004) searched for the identity of the protease(s) which regulate(s) ENaC activity in immortalized JME/CF15 cultures derived from ΔF508 nasal epithelia. These cultures lost protease regulation of ENaC after inhibition of CAP1 activity with siRNA. CAP1 appears to be expressed at much lower level in our cultures than CAPs 2 and 3, perhaps suggesting that multiple CAPs may operate in airway epithelia.

The activity of ENaC, which appears to be at least partially governed by the balance of CAPs and CAP-inhibitors in the ASL, has important consequences for Cl^- secretion via CFTR. ASL $[Cl^-]$ is 3-4 fold higher in the ASL than intracellularly and to permit Cl^- secretion into the ASL, the apical membrane must be hyperpolarized by the inactivation of ENaC, which in turn provides the necessary electrical driving force for cellular Cl^- exit into the ASL (Boucher, 1994). To test this experimentally, we pretreated airway cultures with either trypsin (to maximally activate ENaC), or with aprotinin (to inhibit ENaC) (Fig. 2C-F). As would be predicted, trypsin pretreatment completely blocked adenosine-induced ASL secretion, likely due to the lack of a suitable electrical gradient for Cl^- secretion (Fig. 2E). In contrast, adenosine elicited a robust response following aprotinin pretreatment (Fig. 2E). CF cultures lack the ability to secrete ASL following adenosine addition and ENaC may even be further stimulated by the adenosine-dependent rise in cAMP (Fig. 2F; Stutts et al., 1995). Further, aprotinin addition preserved ASL volume on CF airway surfaces over this period (Fig. 2F; Tarran et al., 2006b), providing further evidence that inhibition of ENaC may be therapeutically relevant in the treatment of CF lung disease.

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How is ASL Volume Sensed by Airway Epithelia?

It is not clear how airway epithelia sense ASL volume and adjust ion transport rates accordingly. Based on our data showing that normal airway cultures set ASL height at ~7 μm, which is the height of outstretched cilia, we hypothesized that cilia may have been acting in a mechanosensory capacity to signal ASL volume status to the underlying epithelia (Figs. 1 & 2). However, we have found no role for cilia in mechanically sensing ASL volume under the conditions tested in Fig. 2 (i.e. static culture conditions following the addition of small amounts of Ringer solution). For example, cultures which have immotile cilia (i.e cultures from donors with primary ciliary diskinesia) and poorly-differentiated cultures with no cilia were still able to effect ASL volume regulation (Tarran et al., 2006b). Thus, we proposed that a series of soluble "reporter molecules" are present in the ASL that are sampled by chemosensors on the extracellular face of the apical membrane to regulate the balance between absorption and secretion (Fig. 3). Likely candidates in normal airway epithelia include, but are not limited to, adenosine, which regulates CFTR via stimulation of the A2B subtype of adenosine receptors (Huang et al., 2001) as well as cell attached channel-activating proteases (CAPs) that activate ENaC (Rossier, 2004). Following small volumes of Ringer solution (i.e. ~20 μl) and in the absence of adenosine/CAP inhibitors, CFTR is quiescent due to the lack of stimulation by adenosine and ENaC activity is close to maximal due to the absence of CAP-inhibitors (Fig. 3A). However, as excess liquid is absorbed over time, adenosine and CAP-inhibitors accumulate and begin to (i) activate CFTR and (ii) inactivate ENaC (Fig. 3B). As this absorptive process continues, ASL will reach a height of ~ 7 μm (Fig. 3B).

Thus, it is likely that the concentration of adenosine and CAP-inhibitors in the ASL are the signals that maintain an appropriate balance between absorption and secretion. Other investigators have shown that adenosine is both broken down to inosine and recycled (Szkotak et al., 2001), so it is likely that in the absence of any acute changes in ASL volume that ASL adenosine is also maintained at a set value rather than accumulating in the ASL. Whether CAPs/CAP inhibitors are similarly regulated is unknown although Verghesse et al (Verghese et al., 2006) have recently demonstrated that CAP1 (prostasin) can be cleaved from the cell surface by GPI-specific phospholipase D1, an enzyme know to be expressed in airway epithelia, suggesting that this system is also regulated. Excess protein may be removed by mucus clearance, again keeping a constant level of CAPs/CAP inhibitors on airway surfaces *in vivo*.

After a steady state ASL height has been reached, the addition of bumetanide, which blocks basolateral Cl^- uptake will induce ASL depletion (Fig. 3C). Similarly, nystatin addition bypasses the physiological inhibition of ENaC and reintroduces Na^+ absorption, also causing ASL collapse (Wu et al., 1998;Tarran et al., 2006b; Fig. 3D).

Under high volume conditions, CF and normal cultures function similarly, i.e. Na^+ absorption through ENaC dominates (Figs. 3A & 4A). However, the rate of Na^+ absorption in CF tissues is greater as a result of Na^+ hyperabsorption through ENaC caused by the lack of CFTR (Boucher et al., 1986). Due to the absence of an appropriate effector (CFTR), CF airways cannot respond to the low ASL volume by initiating secretion (Fig. 4B). For reasons unknown, the CAP mediated activation of ENaC also persists in CF airways and taken together, these abnormalities lead to a volume-depleted ASL which is incompatible with efficient mucus transport. Since CF cultures already lack the ability to secrete or to initiate ENaC autoregulation, bumetanide and nystatin addition are without effect (Tarran et al., 2006b; Fig. 4C, D).

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ASL Regulation under phasic shear stress conditions

CF airway cultures lose the ability to transport mucus within 24-48 h (Matsui et al., 1998), yet it takes months to years for mucus plugs to form *in vivo*, suggesting that aspects of CF ASL volume regulation were missing from our standard, static culture system (Moskowitz et al., 2005). We speculated that phasic motion, similar to the phasic stresses induced by tidal breathing *in vivo*, was missing from standard static culture systems which may provide additional factors that regulate ASL height. Thus, we constructed a novel device that exposed normal & CF cultures to physiologic levels of intermittent shear stress for extended periods (28 episodes of 0.5 dynes.cm^{-2} shear stress every minute to mimic 14 inspirations and 14 expirations per minute). With 48 h of this phasic motion, normal cultures approximately doubled ASL height to 14 μm (Fig 4A, B). Importantly, CF cultures increased ASL height to ~7 μm (Fig 4A, B). This increase in CF ASL height was accompanied by the appearance of Cl^- secretion (Tarran et al., 2005), suggesting that shear stress had activated pathways for Cl^- secretion in CF cultures which are absent under static conditions.

Shear stress has multiple effects on cell function. One almost universal mechanism to couple shear stress to autocrine, and indeed paracrine effects is via the regulated release of ATP into the extracellular environment. Similar to other cell types [e.g., endothelia; (Burnstock, 2006)], cultured airway epithelia continually release purines and increase their rate of ATP release whenever the mucosal media is disturbed (Lazarowski et al., 2004), suggesting that culturing airway epithelia under motion would alter the mucosal purine concentrations. It appears that ATP is released from normal and CF airway epithelia at equal rates (Watt et al., 1998), and that normal & CF ATP concentrations *in vivo* are not different (Donaldson et al., 2000).

Under thin film conditions, we found that phasic shear stress caused a dose-dependent increase in ASL ATP levels in primary cultures of bronchial surface epithelial cells (Tarran et al., 2005). Similarly, Guyot & Hanrahan (Guyot and Hanrahan, 2002) grew CALU3 cells (immortalized cells of glandular origin) in hollow biofibers and found that increasing the flow rate inside the lumen of the fibers caused a rapid, reversible increase in luminal ATP secretion.

That Cl^- secretion is required for increasing ASL height in response to shear stress was demonstrated by the addition of Inh_{172}, a relatively specific CFTR inhibitor (Ma et al., 2002) that decreased ASL height from ~14 to ~7 μm in normal cultures but was without effect in CF cultures (Fig. 5C and (Tarran et al., 2005)), consistent with CFTR being the major pathway for Cl^- secretion in normal airways. In contrast, addition of DIDS, which inhibits CaCC, but not CFTR, further reduced ASL height to ~5 μm in both normal and CF cultures. These data suggest that CaCC is operating in response to shear stress in both normal and CF cultures, and that stimulation of CaCC does not appear to be able to induce the same degree of liquid secretion as stimulation of CFTR. This may be due to the duration of the signal: upon stimulation with cAMP, CFTR remains active as long as the agonist is present. The Ca^{2+} signal is transient and declines within minutes following a single stimulation with nucleotides (Mason et al., 1991). Studies of $P2Y_2$-R have tended to focus on relatively large doses of nucleotides in the μM range, which also induce desensitization of the $P2Y_2$-R. It will be interesting to see whether repeated $P2Y_2$-R-stimulation with sub μM levels of ATP or UTP also cause desensitization or whether a steady state is reached during physiological stimulation of $P2Y_2$-R where the desensitization/removal *vs.* sensitization/insertion level of receptor turnover are matched allowing the epithelia to continually respond to shear stress via $P2Y_2$-R.

Thus, from a basal ASL height of ~ 7 μm (Fig. 6A), normal cultures increase ASL height to ~14 μm upon exposure to phasic shear stress. This effect is mediated in part by increased ATP-dependent activation of $P2Y_2$-R which stimulates CaCC to secrete Cl^-. ATP is also degraded

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to adenosine which also increases activation of A2B-R to further stimulate CFTR (Fig. 6B). In the absence of shear stress, CF cultures have little or no Cl^- secretion and exhibit Na^+ hyperabsorption which combined result in a volume-depleted ASL (Fig. 6C). Following phasic shear stress, ASL ATP levels rise sufficiently to stimulate $P2Y_2$-R and CaCC to increase ASL volume to 7 μm. This height is approximately half of the increase seen in normal airways, reflecting the lack of a response from CF airways to adenosine on $A2_B$-R and CFTR. We have shown that phasic shear stress causes inactivation of Na^+ absorption in CF airway cultures (Tarran et al., 2005). It is unknown whether this inhibition is due to $P2Y_2$-R, which has been shown to inactivate ENaC by depleting PIP2, a molecule whose presence is required for ENaC activation. Also, it is possible that shear stress induces activation of the CAP-inhibitors that are active in normal cultures but inactive in CF cultures under static conditions (Fig. 6D).

Hypertonic saline and CF

It has been proposed that inhaled hypertonic saline (HS), which osmotically draws water from epithelial cells/interstitium into the airway lumen may be used as a therapy to restore ASL volume to CF airways to restore mucus clearance and improve pulmonary function.

The addition of HS to airway epithelia has been tested experimentally using the same airway culture system interfaced to a confocal microscope which is used to understand the physiologic regulation of ASL height (e.g. Figs. 1, 2, 4). NaCl suspended in perfluorocarbon (PFC) has been added to culture surfaces to mimic aerosol delivery of HS *in vivo* (Tarran et al., 2001). This results in a rapid increase in ASL height in normal airway cultures that has only a modest duration of action (Fig. 7;Tarran et al., 2001b;Donaldson et al., 2006). Surprisingly, the same maneuver resulted in a larger and more sustained increase in ASL height in CF airway cultures (Fig. 7;Donaldson et al., 2006) which at first inspection appears at odds with the data and prevailing theory that CF airways hyperabsorb ASL. It is important to understand that HS probes the passive permeability properties of the epithelium in a non-physiological setting, and its actions should not be confused with physiologic active ion transport modes. This difference can be ascribed to the absence of a Cl^- conductance in CF airways and can be mimicked in normal airway cultures by pretreating them with the CFTR inhibitor Inh_{172} or by adding NaGluconate in place of NaCl since CFTR is impermeant to gluconate (Gray et al., 1993).

These differences in absorption rates can be explained using the model shown in Fig. 8. Beginning with normal airway epithelia under baseline conditions, when ASL Na^+ and Cl^- concentrations are isotonic, Na^+ is absorbed through ENaC in response to the apical membrane-cellular electrochemical gradient favoring Na^+ entry (Willumsen and Boucher, 1991a,b). Despite the fact that there is a partially active CFTR Cl^- channel in the apical membrane of the normal airway epithelium, Cl^- is not absorbed with Na^+ transcellularly because there is no electrochemical driving force for Cl^- movement across the cell membrane (Willumsen and Boucher, 1989;Willumsen et al., 1989a,b). Isotope flux and ion-selective electrode data suggest that the overall ion conductance of the cell path is ~2-fold greater than through the paracellular path in normal airway epithelia (Knowles et al., 1983b).

In normal airways, the increase in Na^+ concentration following HS deposition increases the electrochemical driving force for both transcellular and paracellualar Na^+ movement (Fig. 8B). Importantly, the increase in the Cl^- concentration in ASL now generates an electrochemical driving force for Cl^- to enter the cell via CFTR, with the residual Cl^- moving through the paracellular path. The Na^+ and Cl^- that enters the cell in response to the HS-induced NaCl concentration gradient on the airway surface exits the cell through the basolateral Na^{+}-K^{+}-ATPase, a basolateral Cl^- channel and the Na^+-K^+-$2Cl^-$ cotransporter. Interestingly, whilst basolateral Cl^- channels have been reported (Willumsen et al., 1989a,b;Fischer et al., 2007) their molecular identity remains to be determined. Based on the estimates of the relative

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permeabilities of the cell and paracellular paths for Na^+ and Cl^-, it is estimated that ~2/3 of the NaCl may move through the transcellular path in response to these imposed chemical gradients (Knowles et al., 1983b). Note that the HS-induced increase in NaCl concentration on airway surfaces produces a gradient for water to move transepithelially (i.e. via the paracellular pathway and cellular aquaporins) and in the opposite direction to that generated by active ion transport, i.e., there is osmotically induced water flow from the submucosa to the airway surface. We have previously shown that water flux continues for ~30-40 s following a hyperosmotic challenge (Matsui et al., 2000) and during this period of equilibration, NaCl is absorbed in the opposite direction through both the cellular and the paracellular pathways. Thus, due to NaCl absorption after HS-addition, the subsequent osmotic gradient dissipates relatively rapidly and only a modest amount of water moves to airway surfaces in response to this challenge (see Fig. 8C). This additional ASL is then absorbed isotonically, as shown in Fig. 3 A-C since any endogenous "reporter molecules" such as adenosine will have been diluted by the sudden increase in ASL volume (Fig. 8D).

For CF airway epithelia there is an electrochemical driving force for Cl^- to be absorbed across the apical membrane (Knowles et al., 1983b). However, because of the absence of the CFTR Cl^- channel, Cl^- must move through the paracellular path to accompany the transcellular Na^+ absorption. The driving force that serves to match the rate of transepithelial Cl^- absorption with Na^+ absorption is the relatively high transepithelial potential difference (-30 mV, lumen negative) generated by high rate of transcellular Na^+ absorption. The absence of CFTR function in CF means that the paracellular movement of Cl^- becomes rate limiting for liquid absorption which results in an increase in ASL.

When HS (7%) has been used to increase the NaCl concentration on CF airway surfaces, differences in the passive ion permeabilities of CF vs. normal airways are revealed. As for normal airway epithelia, when a high NaCl concentration in the ASL is generated by HS addition, an increase in the electrochemical gradient for Na^+ entry into the cell is developed (Fig. 8E). In CF, even though an increased electrochemical gradient for Cl^- entry is also generated, Cl^- cannot enter the cell due to the absence the CFTR Cl^- channel in the apical membrane and the only available path for Cl^- to be absorbed across the CF airway epithelium, in response to HS via aerosols in ASL, is the paracellular path (Fig. 8E). This lack of Cl^- entry retards transcellular Na^+, since Na^+ is retained in the ASL to preserve electroneutrality.

Data from radioisotopic and electrophysiologic techniques suggest that the paracellular path permeability to NaCl is not different in CF vs. normal airway epithelial cells (Boucher et al., 1986; Willumsen and Boucher, 1989; Willumsen et al., 1989a, b). Therefore, because CF airway epithelia can absorb the aerosol-deposited NaCl only via the paracellular path, the rate of absorption/dissipation of the salt gradient will be reduced by ~60-70% as compared to normal airway epithelia, reflecting the absence of the more permeable cellular route for Cl^- absorption in CF airway epithelia. The slower rate of NaCl absorption leads to a relative retention of NaCl on CF airway surfaces. Thus, the osmotic gradient favoring water flow generated by the aerosolized NaCl persists for longer periods of time in CF than normal airway epithelia, leading to increased volumes of water on CF as compared to normal epithelial surfaces (see Fig. 3D). ASL is then absorbed isotonically, but at a greater rate than normal airways. Since the starting ASL volume was greater than for normal cultures post-HS-addition (Fig. 8C vs. 8F), the time to return to baseline is much greater for CF cultures. Thus, CF airways actually get a "break" after HS-addition in the form of a prolonged duration of the action of HS as compared to normal airways.

With the large NaCl concentration gradients (10's mM – 100's mM) generated by HS deposition on CF airway surfaces, the Cl^- conductance of the paracellular path is limiting for Na^+ and, hence, net NaCl absorption. Under baseline conditions where CF active ion transport

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determines ASL volume, active Na^+ transport generates μM gradients across CF airway epithelia. At these transport rates, the Cl^- conductance of the paracellular path is not limiting, so the rate of active Na^+ transport determines ASL volume. These considerations resolve the apparent paradox between the importance of the absent cellular CFTR Cl^- conductance in the response of the CF airway to aerosolized HS vs. the relative unimportance of the cellular Cl^- conductance in the abnormal NaCl absorptive states that are a feature of CF airways under basal conditions.

Implications for CF Airway Disease

There are several therapeutic maneuvers available that are designed to increase CF airways hydration to enhance mucus clearance. As described above, the use of aerosolized hypertonic saline has shown promise and it has recently been reported that the inhalation of HS (7%, delivered by a jet nebulizer) by CF patients over a two week period increases the rates of mucociliary clearance and improves pulmonary function tests, and, over a one year interval, reduces exacerbations and improves the quality of life (Donaldson et al., 2006; Elkins and Bye, 2006). Importantly, the inhalation of HS for one year was not associated with any adverse events such as increased inflammation and/or increased bacterial colonization (Elkins and Bye, 2006).

INS37217 activates apical membrane $P2Y_2$ receptors to stimulate Cl^- secretion via CaCC and to inhibit ENaC which is also predicted to be beneficial in promoting CF airway secretions (Yerxa et al., 2002). However, exercise is also important in promoting mucus clearance and slowing the progression of CF lung disease (Schneiderman-Walker et al., 2000; Bradley and Moran, 2002). Exercise likely operates by the same pathway, i.e. modulation of the $P2Y_2$ response to increase ASL secretion. Recent studies have suggested that post-exercise, the Na^+ transport path in CF airway epithelia is inhibited (Alsuwaidan et al., 1994; Hebestreit et al., 2001). Our data, showing that phasic motion induces ATP release and that ATP inhibits Na^+ transport and initiates Cl^- secretion (Tarran et al., 2005), provide a mechanism to account for amelioration of the severity of CF lung disease by exercise. Thus, we would stress the importance of regular exercise programs in CF to promote a favorable balance of ion transport and facilitate mucus clearance.

Conclusions

In normal airways, the concentration of reporter molecules such as nucleotides/nucleosides and CAPs/CAP-inhibitors within the ASL modulates the relative rates of Na^+ absorption and Cl^- secretion to maintain ASL volume at levels adequate for mucus transport under basal conditions and following periods of shear stress of varying intensity. In CF airways, ASL volume regulation is severely limited by the absence of CFTR-mediated Cl^- secretion and a failure to regulate Na^+ transport. Our data demonstrate that CF airway epithelia can maintain some ASL and mucus transport under conditions of phasic motion that promote ATP release by utilizing the “reserve” $P2Y_2$/CaCC system to secrete Cl^-. However, the CAP system, which appears to play a major role in regulating ASL volume, is defective in CF airways. It is not clear whether CAP activity is also altered under phasic motion conditions, nor how it is influenced by the lack of CFTR. In summary, our studies point to the central role of “chemosensing” in ASL regulation in health and disease. A better understanding of how this chemosensing mechanism operates under all conditions experienced by the lung will be of value in understanding and treating chronic lung diseases typified by mucus hypersecretion such as CF.

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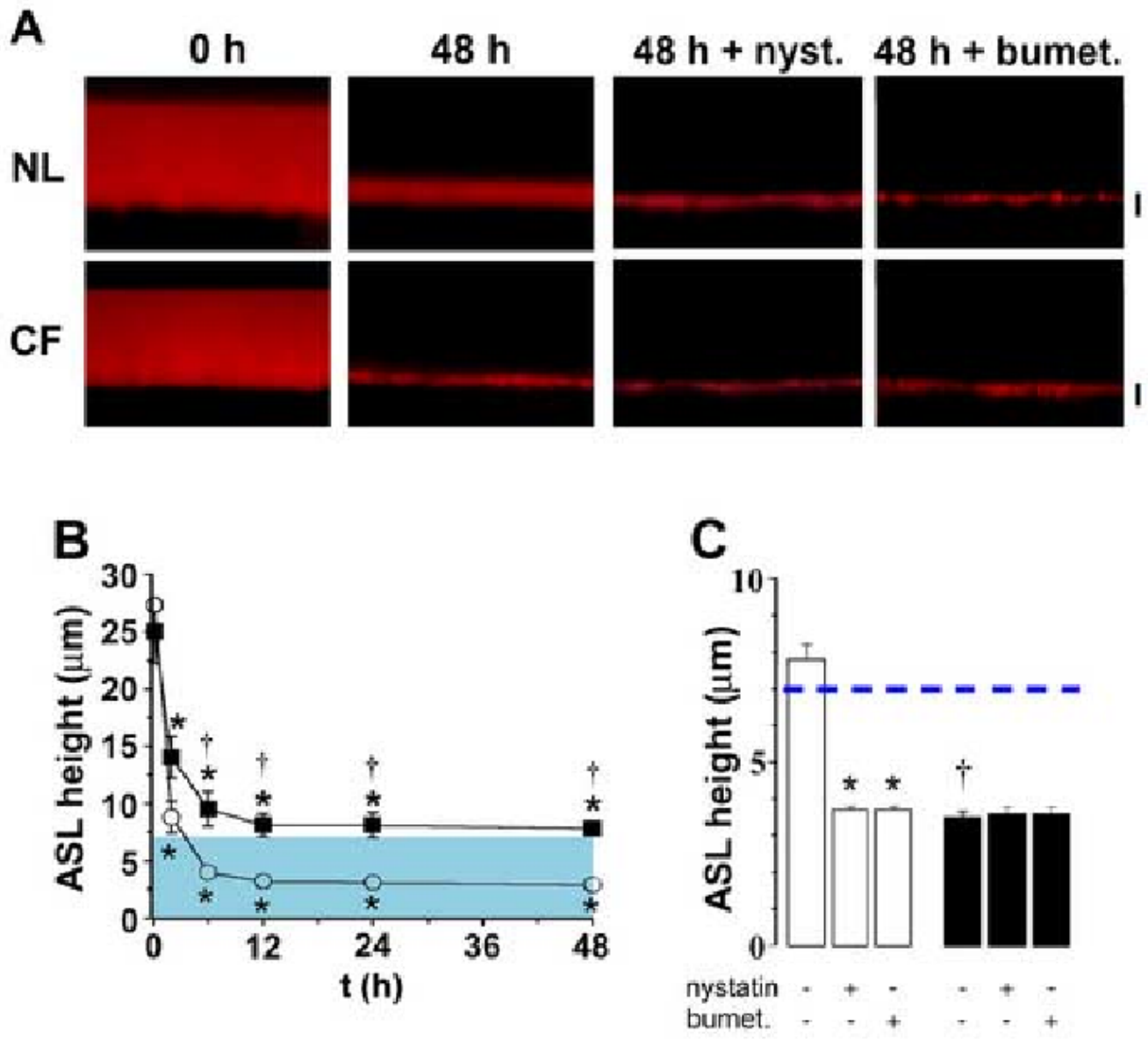

Figure 1. Regulation of ASL height requires a balancing of Na^+ absorption and Cl^- secretion

A, XZ confocal images of ASL labeled with Texas red-dextran. **B**, mean ASL height taken with time under control conditions, i.e. following the addition of 20 μl PBS labeled with Texas Red dextran. **C**, ASL height measured after 48 h under control conditions, and following addition of 100 μM serosal bumetanide or 10 μM mucosal nystatin to inhibit Cl^- secretion or activate Na^+ absorption respectively. Data shown as mean ± standard error. * $p \leq 0.05$ different to control. † $p \leq 0.05$ different between normal & CF.

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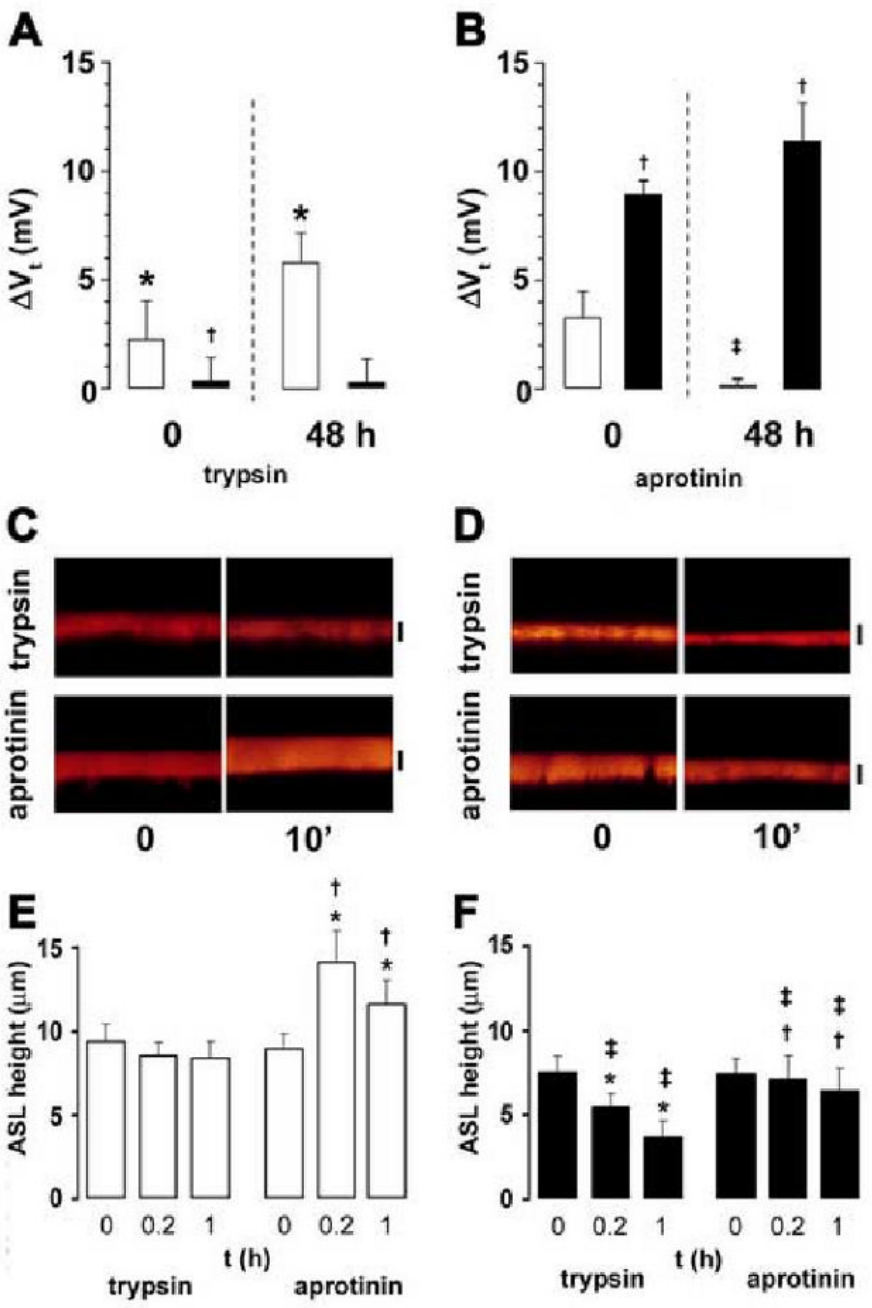

Figure 2. Interaction between protease and adenosine pathways for ASL regulation

A, Change in transepithelial electric potential difference (V_t) across normal (open bars) and CF cultures (closed bars) after 30 min apical trypsin exposure (1.5 U/ml) at 0 or 48 h after PBS (20 μl) addition (all n = 10 and 6 for normal, respectively; and 11 and 8 for CF, respectively). **B**, Change in V_t after 30 min apical aprotinin exposure (2 U/ml) to normal (open bars) or CF (closed bars) cultures at t = 0 or 48 h after PBS addition (n = 5 and 5 for normals; 4 and 4 for CFs, respectively). *N.B., all significant changes in V_t were abolished by amiloride pretreatment* (3×10^{-4} *M; all n = 4*). **C & D**, XZ confocal images of normal & CF cultures respectively acutely prewashed with PBS containing Texas red-dextran and either trypsin (1.5 U/ml) or aprotinin (2 U/ml) 0, 10 and 60 min post-adenosine addition (300 μM). **E& F**, mean

data taken from C & D. Open bars, normal cultures. Closed bars, CF cultures. All data points are n = 6. * Different ($p < 0.05$) from t=0. † Different ($p < 0.05$) from equivalent time point in the presence of trypsin. ‡ Different ($p < 0.05$) between normal and CF cultures. Scale bars are 7 μm.

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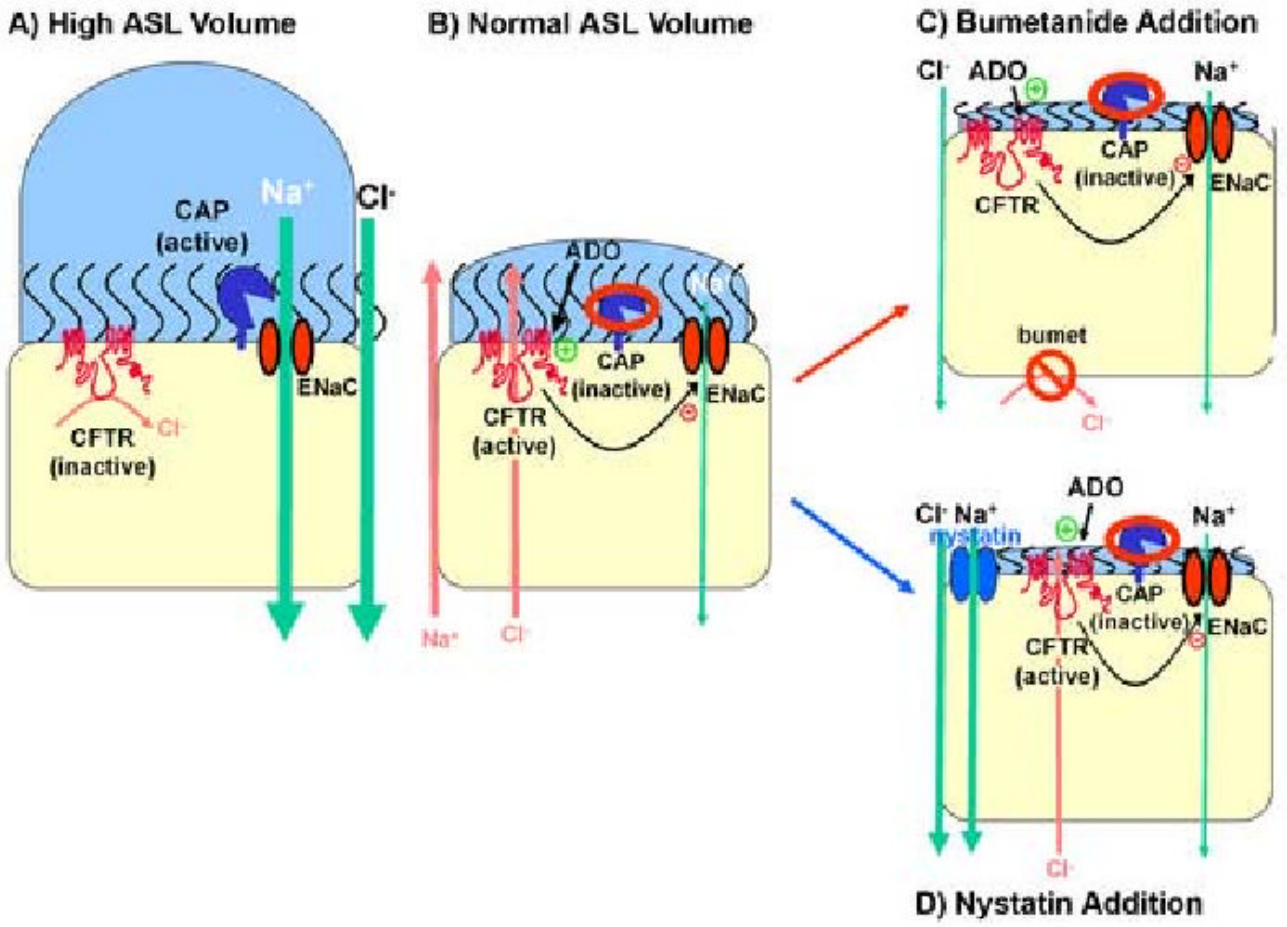

Figure 3. Cartoon depicting changes occurring during Normal ASL volume regulation under static conditions and how this process may be probed pharmacologically

A, in normal airways under high volume conditions, any soluble regulatory molecules such as adenosine (ADO) or secreted protease inhibitors are diluted to such an extent that CFTR is inactive and anion secretion does not occur and ENaC is near to fully active, leading to Na^+-led isotonic ASL absorption with Cl^- following through the paracellular pathway. **B**, as ASL volume diminishes, adenosine accumulate in the ASL sufficiently to activate CFTR (likely by stimulation of A2B adenosine receptors) and protease inhibitors (red circles) are secreted to mostly inactivate ENaC via inhibition of channel-activating proteases (CAPS). This leads to a steady state ASL height which approximates the height of outstretched cilia (7 μm) which is maintained by continued anion secretion through CFTR that is offset by a moderate amount of Na^+ absorption through ENaC. **C**, to confirm that steady state ASL height is maintained by active ion transport, bumetanide can be applied serosally to block Cl^- entry into the epithelia which is predicted to result in ASL collapse to CF levels (i.e. 3-4 μm). *N.B., ASL HCO_3^- (< 10 mM) is insufficient to maintain a suitable ASL height in the absence of Cl^-secretion*. **D**, the physiological inhibition of ENaC with time is also required for maintenance of a steady-state ASL height and addition of a cationophore (nystatin) allows unregulated Na^+-led ASL absorption to occur which is also predicted to cause ASL collapse.

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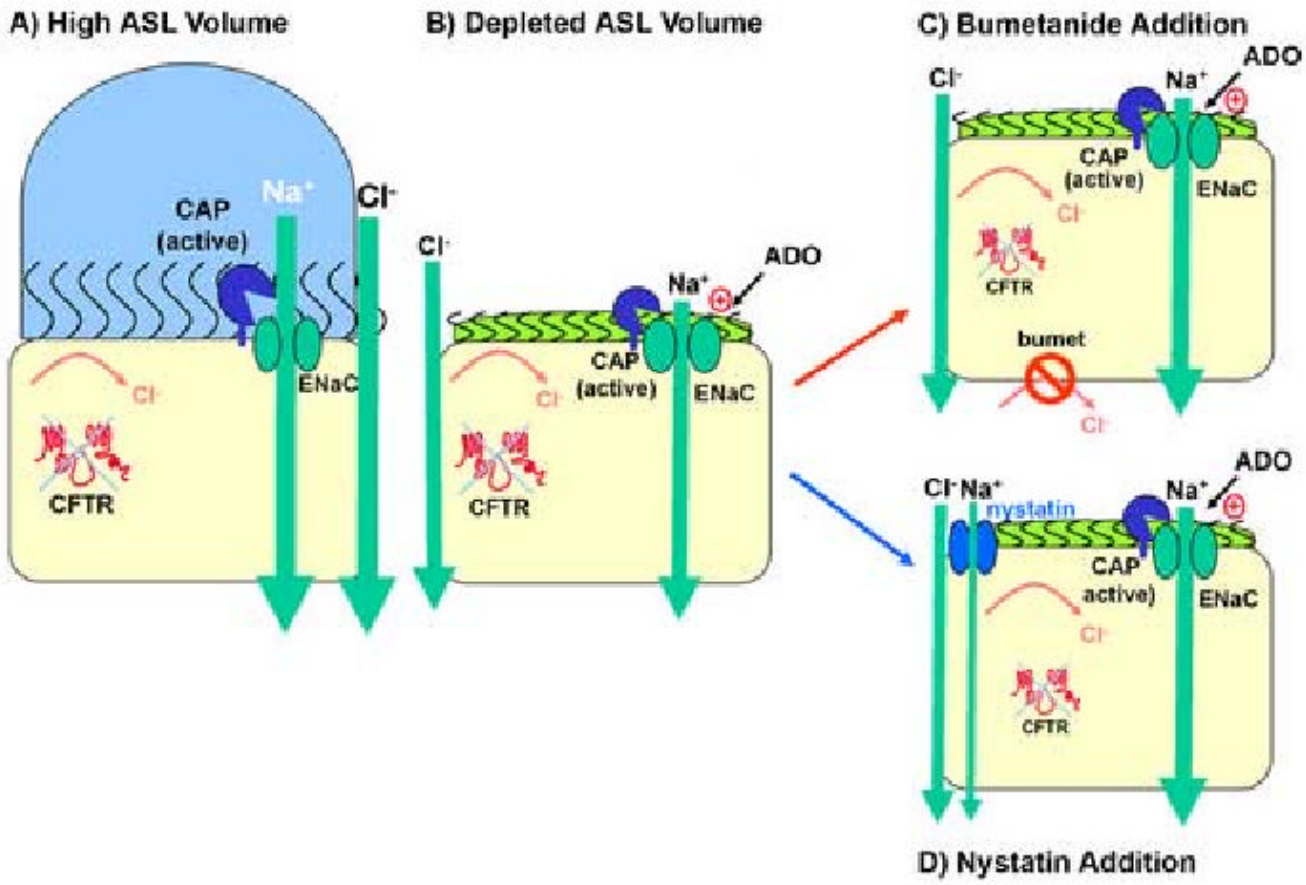

Figure 4. Cartoon showing how abnormal Cl^- secretion and Na^+ absorption in CF airways under static conditions lead to ASL volume depletion

A, in CF airways under high volume conditions, as in normal airways, any soluble regulatory molecules such as adenosine or secreted protease inhibitors are diluted and ineffective. Due to the lack of CFTR and its inhibitory effects on ENaC, ENaC is hyperactive leading to a more rapid baseline Na^+ led volume absorption than in NL airways under high volume conditions with Cl^- following through the paracellular pathway. **B**, as ASL volume is reduced, regulatory molecules such as ADO and CAP-inhibitors accumulate in the ASL. However, in the absence of CFTR activation of A2B-R is predicted to raise cAMP and stimulate rather than inactivate ENaC. Further, for reasons that are currently unknown, the CAP system is also dysfunctional and ENaC remains proteolytically cleaved despite a reduction in ASL volume. Thus, unlike NL airways, CF airways are unable to make the switch from an absorbing to a secreting epithelia and ASL volume depletion results. **C**, **D**, unlike NL airways, since ASL volume is already depleted, bumetanide and nystatin addition are without affect. Note, since ASL volume does not fall below ~3 μm in airway epithelia, transcellular Na^+ absorption will likely be offset by a backflux of Na^+ through the paracellular pathway.

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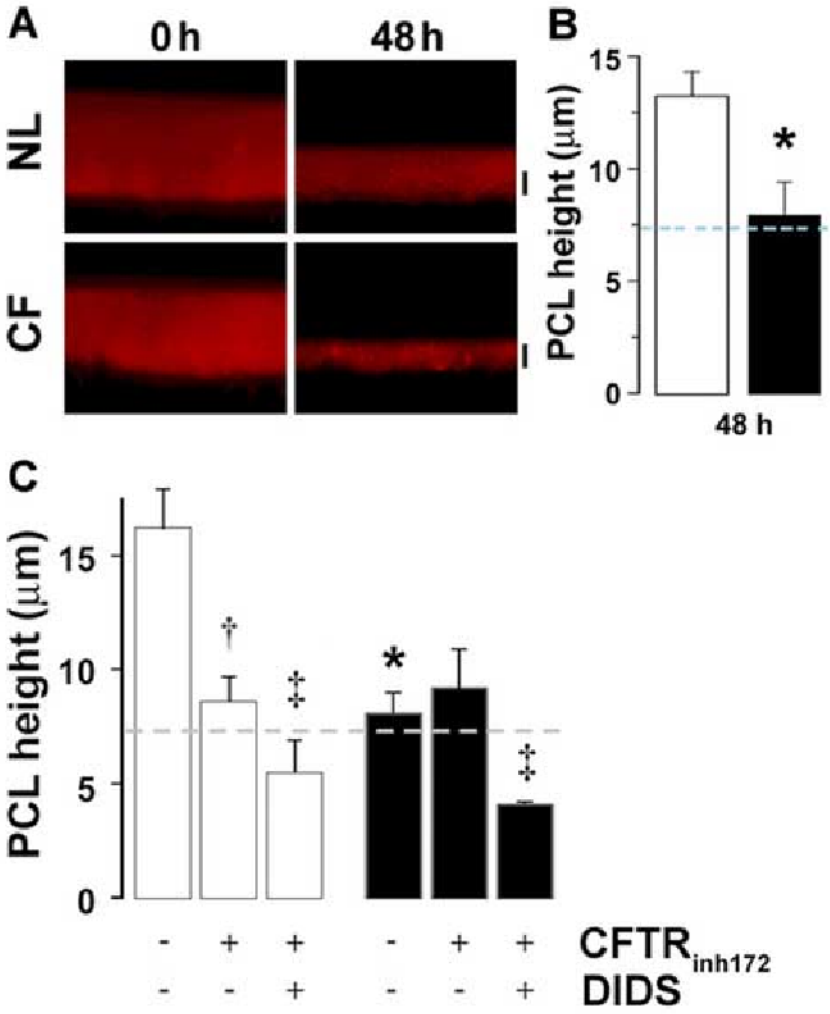

Figure 5. Phasic motion-induced changes in PCL volume in normal vs. CF cultures

A, XZ confocal images of PCL immediately (0) and 48 h after mucosal PBS addition to normal and CF epithelia cultured under phasic motion. **B**, Mean PCL heights after 48 h of phasic motion culture for normal (open bars, n = 7) and CF (closed bars, n = 8). **C**,. Mean PCL height after 3 h of phasic motion in the presence of a CFTR antagonist ($CFTR_{inh172}$; 10 μM) or $CFTR_{inh172}$ and a CaCC antagonist (DIDS; 100 μM). Normals (open bars; n = 6) and CFs (closed bars; n = 6). Data shown as mean ± S.E.M. *Data significantly different between normal and CF cultures. † Data significantly different from control. ‡Significantly different between static and phasic motion or significantly different from $CFTR_{inh172}$.

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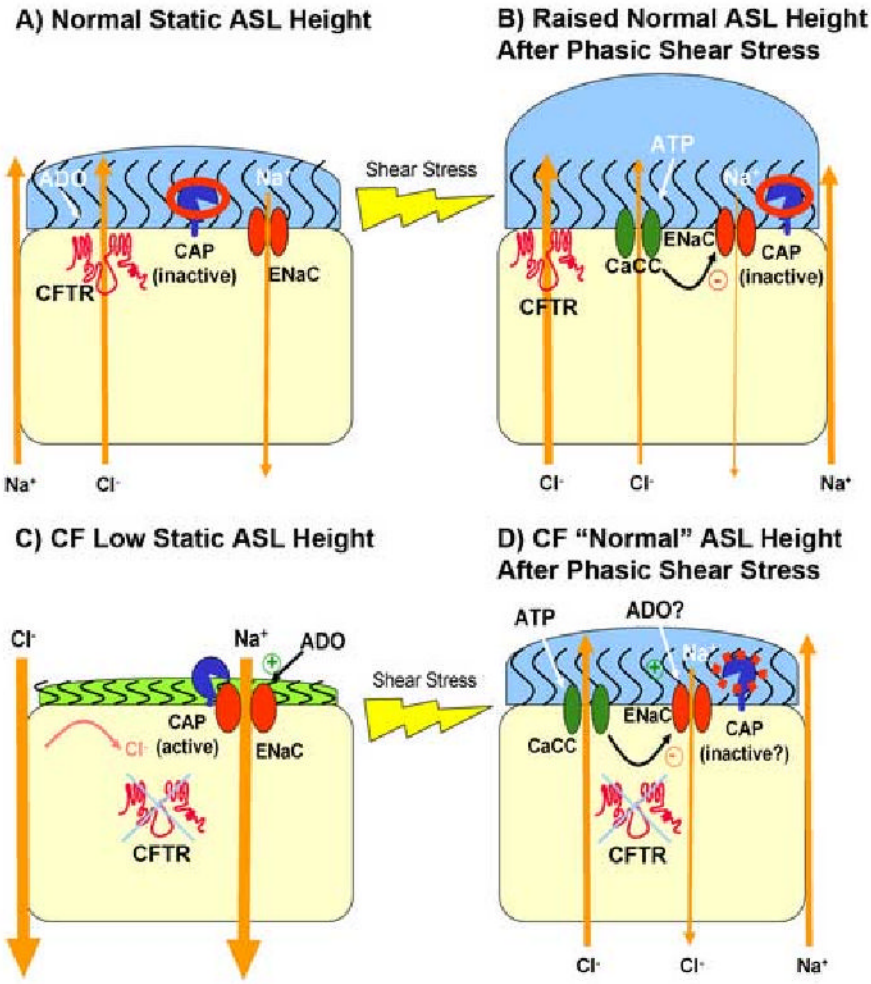

Figure 6. Schema describing ASL height regulation by phasic shear stress

A, Normal airway epithelia under static conditions coordinately regulate the rates of Na^+ absorption and Cl^- secretion to set ASL volume at 7 μm. **B**, Normal airway epithelia under phasic motion respond to increased nucleotide/nucleoside release into the ASL by shifting the balance further towards Cl^- secretion via CFTR and Ca^{2+} activated Cl^- channel (CaCC) resulting in a greater ASL height. **C**, in CF epithelia, the higher basal rate of Na^+ absorption, the failure to inhibit Na^+ transport rates, and the failure to initiate Cl^- secretion under static conditions lead to PCL depletion (note "flattened" cilia). **D**, CF cultures under phasic motion conditions release sufficient ATP into the ASL to inhibit Na^+ absorption and initiate CaCC mediated Cl^- secretion to restore ASL to a physiologically adequate height.

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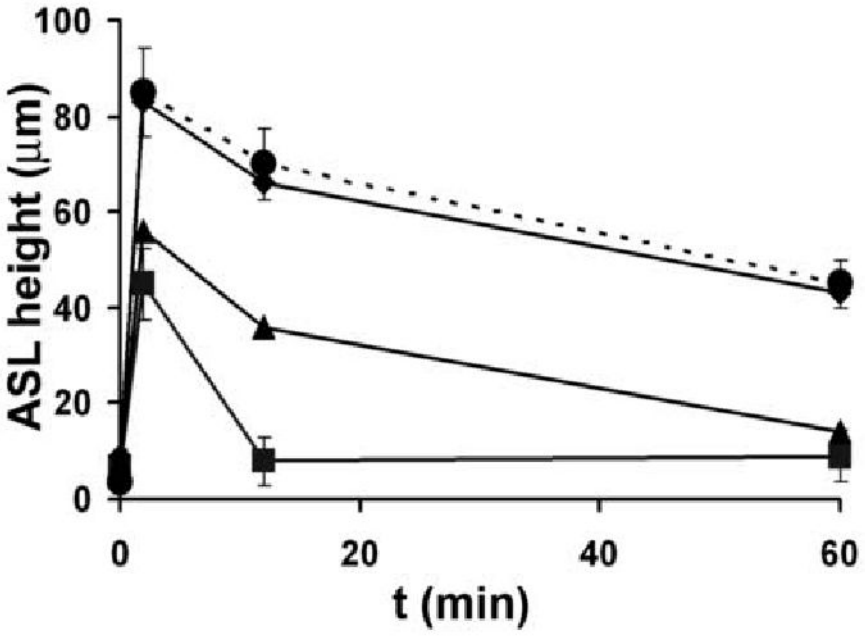

Figure 7. Normal and CF airway epithelia respond differently to hypertonic saline and to amiloride

~ 1000 mM NaCl is delivered to the mucosal surface of well-differentiated normal and CF bronchial cultures and ASL height monitored by XZ confocal microscopy. Normal cultures with NaCl (■), CF cultures with NaCl (●), NaCl and 10 μM Inh_{172} (▲), Na gluconate (◆).

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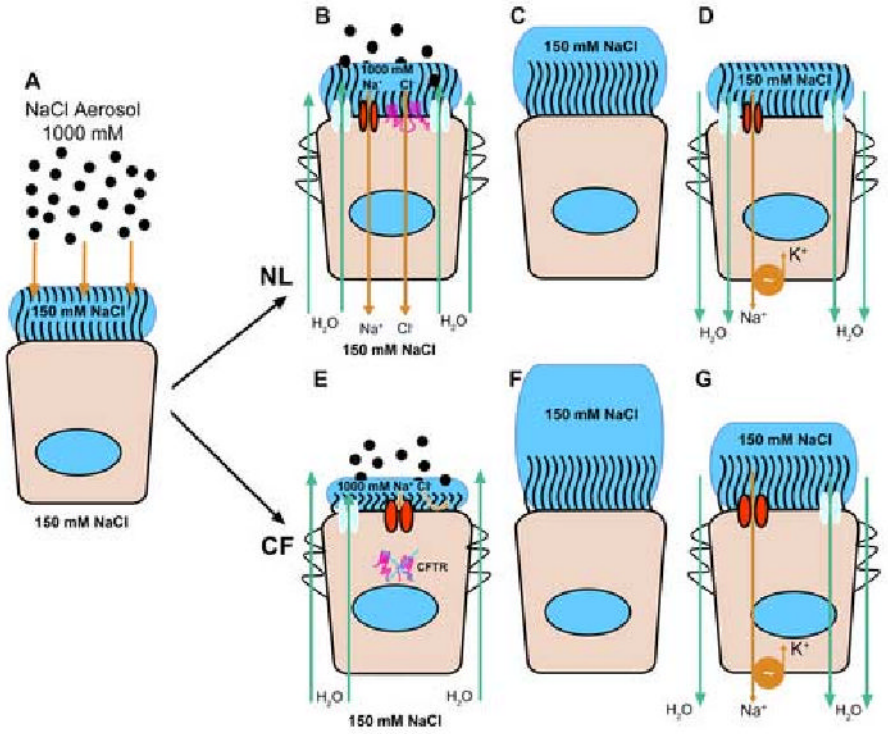

Figure 8. Cartoon predicting a differential response to hypertonic saline addition in normal *vs*. CF airway epithelia

A, 1000 mM NaCl is deposited on airway surfaces following inhalation of nebulized ~6% hypertonic saline. **B**, in NL airways, the increase in the NaCl concentration following HS deposition increases the electrochemical driving force for Na^+ to move transcellularly through ENaC (orange arrows) as well as increasing the electrochemical driving force for Na^+ to move through the paracellular pathway (not shown). Importantly, the unphysiologically large increase in the ASL Cl^- concentration now generates a sufficiently large electrochemical driving force for transcellular Cl^- absorption via CFTR, with the some Cl^- also moving through the paracellular path (~33%; not shown). The Na^+ and Cl^- that enters the cell in response to the HS-induced NaCl concentration gradient on the airway surface exits the cell through the basolateral Na^+-K^+-ATPase, a basolateral Cl^- channel and the Na^+-K^+-2Cl^- cotransporter (not shown). Concomitantly, H_2O moves in the opposite direction from the serosa into the ASL both through aquaporins and paracellularly (green arrows) to increase ASL height, although due to the rapid, passive transcellular absorption of NaCl, the NaCl-dependent increase in ASL height is less than would be predicted if all 1000 mM NaCl remained on airway surfaces. **C**, following this period of rapid equilibration, ASL height is moderately raised as compared to A. **D**, Due to dilution of sensor molecules in the ASL, isotonic Na^+ led absorption returns ASL height to a depth of 7 μm. **E**, even though an increased electrochemical gradient for Cl^- entry is also generated by HS addition in CF airways, Cl^- cannot enter the cell due to the absence of the CFTR Cl^- channel in the apical membrane. Since Cl^- is not absorbed to preserve electroneutrality, this also retards Na^+ absorption (orange arrows). Thus, NaCl absorption must occur in CF airways via the paracellular pathway (not shown) which has only ~33% of the capacity of the transcellular pathway. This results in a smaller dissipation of available osmoles and a larger steady state ASL height **(F). G**, Isotonic Na^+ hyperabsorption results in isonoic removal of this ASL with time.