

## CALL FOR PAPERS | *Ion Channels and Transporters in Lung Function and Disease*

### Role of epithelial sodium channels in the regulation of lung fluid homeostasis

Sadis Matalon,<sup>1,2,3,4</sup> Rafal Bartoszewski,<sup>5</sup> and James F. Collawn<sup>2,3,4</sup>

<sup>1</sup>Department of Anesthesiology and Perioperative Medicine, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; <sup>2</sup>Department of Cell, Developmental, and Integrative Biology, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; <sup>3</sup>Pulmonary Injury and Repair Center, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; <sup>4</sup>Gregory Fleming James Cystic Fibrosis Center, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; and <sup>5</sup>Department of Biology and Pharmaceutical Botany, Medical University of Gdansk, Gdansk, Poland

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**Matalon S, Bartoszewski R, Collawn JF.** Role of epithelial sodium channels in the regulation of lung fluid homeostasis. *Am J Physiol Lung Cell Mol Physiol* 309: L1229–L1238, 2015. First published October 2, 2015; doi:10.1152/ajplung.00319.2015.—In utero, fetal lung epithelial cells actively secrete  $\text{Cl}^-$  ions into the lung air spaces while  $\text{Na}^+$  ions follow passively to maintain electroneutrality. This process, driven by an electrochemical gradient generated by the  $\text{Na}^+/\text{K}^+$ -ATPase, is responsible for the secretion of fetal fluid that is essential for normal lung development. Shortly before birth, a significant upregulation of amiloride-sensitive epithelial channels (ENaCs) on the apical side of the lung epithelial cells results in upregulation of active  $\text{Na}^+$  transport. This process is critical for the reabsorption of fetal lung fluid and the establishment of optimum gas exchange. In the adult lung, active  $\text{Na}^+$  reabsorption across distal lung epithelial cells limits the degree of alveolar edema in patients with acute lung injury and cardiogenic edema.  $\text{Cl}^-$  ions are transported either paracellularly or transcellularly to preserve electroneutrality. An increase in  $\text{Cl}^-$  secretion across the distal lung epithelium has been reported following an acute increase in left atrial pressure and may result in pulmonary edema. In contrast, airway epithelial cells secrete  $\text{Cl}^-$  through apical cystic fibrosis transmembrane conductance regulator and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels and absorb  $\text{Na}^+$ . Thus the coordinated action of  $\text{Cl}^-$  secretion and  $\text{Na}^+$  absorption is essential for maintenance of the volume of epithelial lining fluid that, in turn, maximizes mucociliary clearance and facilitates clearance of bacteria and debris from the lungs. Any factor that interferes with  $\text{Na}^+$  or  $\text{Cl}^-$  transport or dramatically upregulates ENaC activity in airway epithelial cells has been associated with lung diseases such as cystic fibrosis or chronic obstructive lung disease. In this review we focus on the role of the ENaC, the mechanisms involved in ENaC regulation, and how ENaC dysregulation can lead to lung pathology.

oxidative stress; plasminogen activator; steroid hormones; stem cells;  $\beta$ -adrenergic agonists

THE MOST IMPORTANT ION TRANSPORT process of normal alveolar epithelial cells is active transport of  $\text{Na}^+$  from the alveolar lining fluid (ALF) to the interstitium, down an electrochemical gradient created by the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase (5, 94, 98).  $\text{Cl}^-$  ions move passively across paracellular junctions of alveolar epithelial cells or transcellularly to maintain electroneutrality (98). The vectorial movement of  $\text{Na}^+$  and  $\text{Cl}^-$

creates an osmotic gradient, which causes fluid to move passively from the air space to the interstitium [alveolar fluid clearance (AFC)]. This process is crucial for the reabsorption of alveolar edema following alveolar flooding, which occurs when alveolar permeability to fluid and plasma proteins is increased, resulting in more efficient gas exchange. For example, patients with acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) with normal AFC have lower morbidity and mortality than those with compromised AFC (98, 99). Furthermore,  $\text{Na}^+$  transport through epithelial  $\text{Na}^+$  channels (ENaCs) is also essential for the resolution of high-altitude edema (130). In mouse models, inhibition of  $\alpha$ -ENaC (the pore-forming subunit of ENaC) by siRNA in the adult lung decreased basal fluid clearance, as well as terbutaline-stimulated clearance (87). Complete knockout (KO) of  $\alpha$ -ENaC is lethal, because these mice are unable to clear lung fluid at birth (61). Conditions that increase  $\text{Cl}^-$  secretion across alveolar epithelial cells (such as an acute increase in left atrial pressure) may result in pulmonary edema (138). Novel methodologies using imaging techniques to assess  $\text{Na}^+$ -driven AFC have been reviewed recently by Gammon et al. (43).

In contrast, airway epithelial cells absorb  $\text{Na}^+$  and actively secrete  $\text{Cl}^-$ , which enter the basolateral membranes through  $\text{Na}^+/\text{K}^+/\text{2Cl}^-$  transporters (94), are transported down an electrochemical gradient generated by the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase, and exit through apical  $\text{Cl}^-$  channels, including the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) (124) and transmembrane protein 16a (TMEM16A). TMEM16A, a  $\text{Cl}^-$  channel and member of the anoctamin family, is thought to compensate for CFTR in the mouse lung, potentially providing a rationale for the lack of a lung phenotype in CFTR KO mice (126). However, other channels, such as CLCN2 (131), must play a role, since up to 40% of the UTP-stimulated  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel activity persists in TMEM16A KO mice. Interestingly, recent experiments have shown CFTR to play a direct role in  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  transport (126).

The airway surface liquid (ASL), which is composed of the periciliary layer (PCL) and the mucus layer, coats the lining of the airways and functions to trap and expel inhaled pathogens and particulate matter (reviewed in Ref. 89). Under normal conditions, the PCL is  $\sim 7\ \mu\text{m}$  thick; this layer is critical for cilia-mediated transport of the mucus to the larynx, and effective mucociliary transport clears the airway surface of patho-

Address for reprint requests and other correspondence: J. F. Collawn, Dept. of Cell, Developmental and Integrative Biology, Univ. of Alabama at Birmingham, 1918 Univ. Blvd., MCLM 395, Birmingham, AL 35294 (e-mail: jcollawn@uab.edu).

gens and other noxious substances (89). The ENaCs, the CFTR, and  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels work in concert to regulate PCL height, viscosity, and/or pH and, therefore, are critical components of mucociliary clearance (MCC) (Fig. 1) (8, 23, 24, 37, 44, 72, 96, 118, 137, 152). Overexpression of the ENaC  $\beta$ -subunit in mice produces CF-like lung disease because of an excess amount of fluid absorption (93). Compromised MCC leads to chronic bacterial infections and subsequent inflammatory responses in a number of lung diseases, including CF and chronic obstructive pulmonary disease (COPD) (1, 119, 123). The focus of this review is on the role of ENaC in surface hydration (23) and the factors that influence its expression and function, rather than on how changes in pH in the PCL influence mucus viscosity and elasticity due to changes in bicarbonate secretion (16, 25, 30, 118).

#### Oxidative Stress Effects on $\text{Na}^+$ Channel Activity

Oxidative stress is a common component in many lung diseases, including ALI, COPD, asthma, and lung cancer (78, 108, 112, 120, 144, 145). To combat oxidative stress in the lower respiratory tract, a number of antioxidants, including ascorbate, urate, and glutathione (GSH), are present in high levels in the alveolar epithelial lining fluid (9, 13, 59, 74, 86, 140, 149). The redox potential of the GSH-to-oxidized GSH (GSSG) ratio is known to correlate with the ability of the lungs to suppress an oxidative insult (11, 150), and GSSG is increased in the alveolar fluid of patients with ARDS (12). Recently, Downs and colleagues (32, 33) provided a mechanism for why this is a problem. They found that GSSG inhibits ENaC activity in primary alveolar epithelial cells (32, 33). They examined the effect of decreasing the ratio of GSH to GSSG on ENaC activity by examining single-channel recordings from primary alveolar type 1 (AT1) and type 2 (AT2) cells from lung slices or isolated from rat lungs (33). The oxidized redox potentials decreased ENaC activity, and in vivo studies demonstrated compromised fluid clearance in GSSG-instilled mouse lungs. These studies demonstrated that the redox potential of the lung directly affected lung fluid balance (32) through modulation of ENaC activity and suggest that the regulation of the redox potential by antioxidants needs to be tested as a potential therapy, given the demonstrated inhibitory effects of GSSG on ENaC activity. Indeed, antioxidants prevented and reversed  $\text{Cl}^-$ -induced decreases in amiloride-sensitive short-circuit currents across confluent monolayers of rat alveolar epithelial cells mounted in Ussing chambers (79).

#### Cholinergic Regulation of $\text{Na}^+$ channels

Cholinergic receptors in bronchial smooth muscle are well-established targets in COPD and asthma (49, 53, 127). Takemura and colleagues (143) examined the role of cholinergic regulation of ENaC in alveolar epithelial cells. Using single-channel analysis and biochemical approaches, they found that carbachol and oxotremorine activated muscarinic receptors in AT2 cells and activated ENaC in a dose-dependent manner, whereas nicotine did not. This activity could be blocked with atropine, supporting the view that ENaC activity was induced by activation of muscarinic cholinergic receptors in the AT2 cells. Takemura and colleagues also showed that the AT2 cells had muscarinic  $\text{M}_2$  and  $\text{M}_3$  cholinergic receptors and that the cholinergic agonists activated RhoA in these cells. Finally, an

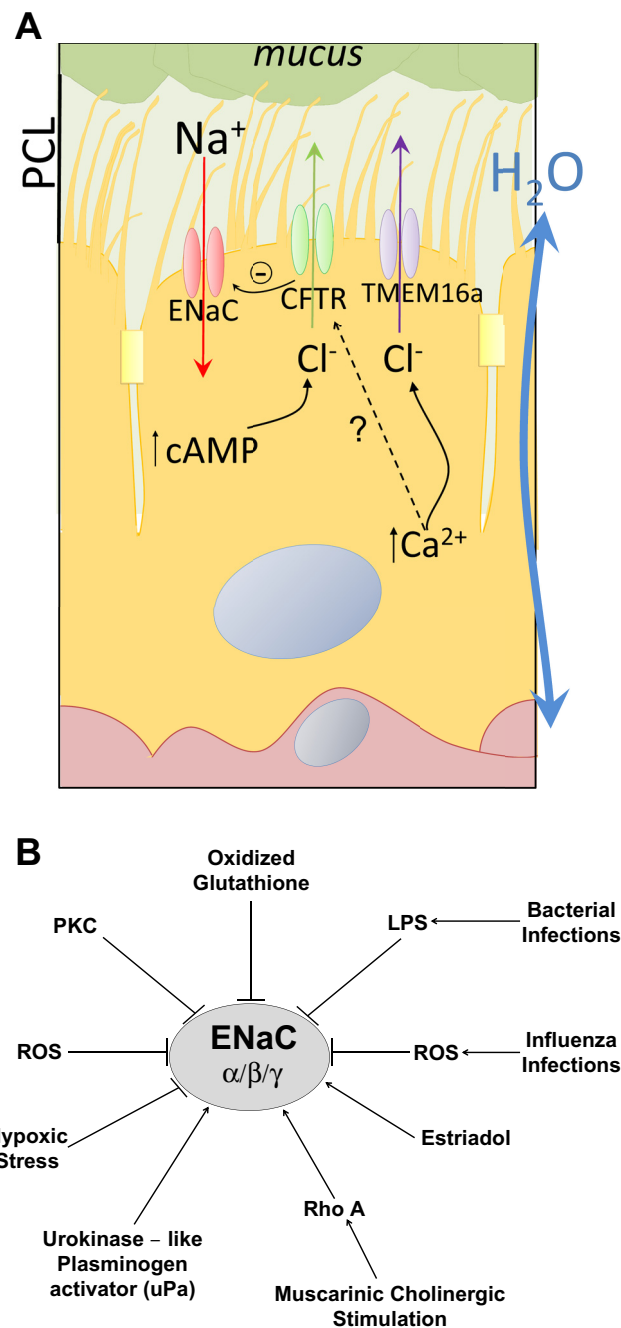


Fig. 1. A: apical  $\text{Na}^+$  and  $\text{Cl}^-$ /bicarbonate channels in alveolar epithelia and their role in regulating airway surface liquid levels in the lung. Three channels in alveolar epithelial cells regulate ion composition in the airway surface liquid. In turn, ion composition regulates the water levels and pH required for efficient gas exchange and mucociliary clearance. Alterations in  $\text{Na}^+$  channel function often lead to a number of lung diseases, and one prominent model, the volume hypothesis, suggests that decreases in cystic fibrosis transmembrane conductance regulator (CFTR) expression and/or function lead to elevated epithelial  $\text{Na}^+$  channel (ENaC) function, increased  $\text{Na}^+$  absorption, and subsequently, lung dehydration and defects in mucociliary transport. This chronic bronchitis phenotype would be illustrated in cystic fibrosis and chronic obstructive pulmonary disease patients and lead to bacterial infections, inflammation, and, eventually, decreases in lung function. PCL, periciliary layer. B: factors that elevate or inhibit ENaC channel expression and/or function in alveolar cells. A number of factors enhance or inhibit ENaC activity, and each of those shown is discussed in this review. LPS, lipopolysaccharide; PKC, protein kinase C; ROS, reactive oxygen species.

inhibitor of Rho-associated protein kinase, Y-27632, was shown to block carbachol-induced activation of ENaC, as well as even basal ENaC activity, suggesting that RhoA activity is essential for this process in rat AT2 cells (143). The connection between RhoA activity and ENaC activity may be in part due to the known effects of RhoA on the cytoskeleton and in promoting ENaC trafficking to the plasma membrane (115). The importance of these studies is highlighted by the fact that, in COPD, where cholinergic regulation is dominant, hypersecretion occurs in the airway, whereas the present results suggest that  $\text{Na}^+$  reabsorption and water in the alveolar space could partially compensate for this effect (143). However, activation of RhoA is known to disrupt lung barrier function (67, 102, 114, 132). These studies emphasize the need to understand the overall regulation of secretion and absorption in the various locations in the lung in different lung pathologies (92).

#### *Plasminogen Activator Effects on $\text{Na}^+$ Channels*

Urokinase-like plasminogen activator (uPA) is a serine protease that converts plasminogen to active plasmin, triggering a proteolysis cascade that promotes fibrinolysis. uPA is expressed in the airways, and its activity is readily detectable in lung lavage fluids of normal animals (62, 97, 136). Perhaps more importantly, the majority of plasminogen activator activity in the lung lavage fluids is from uPA, rather than tissue plasminogen activator (76). Given that fibrinolytic proteases such as uPA have been implicated in a number of lung diseases (129), Chen et al. (21) measured ENaC activity in primary murine tracheal epithelial cells from uPA<sup>-/-</sup> mice. In Ussing chamber experiments, they demonstrated that both basal and cAMP-activated ENaC were reduced in monolayers of tracheal epithelial cells from uPA-deficient mice. The fluid height of the mouse tracheal epithelial cell cultures was more than double that of the uPA<sup>-/-</sup> cells compared with wild-type controls. Causality was demonstrated by the addition of uPA and plasmin to the uPA<sup>-/-</sup> cells, which was able to partially reverse this effect. uPA is known to exert its effects through multiple processes, including the direct proteolysis of the  $\gamma$ -chain of ENaC, regulation of  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity, and modulation of ERK1/2 signaling (7, 60, 83, 113, 135). This study represents the first evidence that uPA upregulates ENaC activity in vitro and in vivo and indicates that alteration of uPA activity could lead to increases in ASL through decreases in ENaC activity. Furthermore, it suggests that changes in uPA activity in injured lungs could directly influence ENaC activity and, therefore, alter the composition or levels of airway fluid, thus further exacerbating lung injury. On the other hand, increased ENaC activity will limit the degree of alveolar edema in ALI (98, 100).

#### *Steroid Hormone Effects on Alveolar Fluid Clearance*

Sex differences show up in many aspects of physiology, including lung pathology (14, 55, 70, 77). A good example is the greater incidence of bacterial lung infections and higher risk of hospitalizations for female than male CF patients (109, 141, 153). Furthermore, this increased susceptibility to severe and often fatal complications from lung influenza infections also appears to be the case in young adult female patients with influenza-associated pneumonia compared with male patients

(71, 133). A common feature of influenza infections and CF is the presence of ENaC dysregulation (20, 23). Given that female patients have poorer outcomes in respiratory disorders, Greenlee and coworkers (52) examined the effect of estradiol on ENaC in rat alveolar cells. Cell-attached patch-clamp analysis was used to monitor single-channel ENaC activity in a rat alveolar cell line [L2, which has an AT2 phenotype (31)] after overnight treatment with estradiol or progesterone. They established that estradiol, but not progesterone, increased ENaC activity by increasing the open probability ( $P_o$ ) and number of channels. Comparison of lung homogenates from female rats in proestrus vs. diestrus revealed that when serum estradiol levels are highest (in proestrus), the plasma membrane levels of ENaC are at their highest. Estradiol primarily affected the activity of the nonselective ENaC channels, and these effects were mediated through the G protein-coupled estrogen receptor (52). Greenlee and coworkers point out an interesting correlation between estradiol and complications in females from influenza-associated pneumonia. In female rats, influenza is known to cause a constant state of diestrus, when the estradiol levels are at their lowest, and treatment of these animals with estradiol attenuates the mortality (125). Greenlee and coworkers propose that low levels of estradiol in female influenza patients lead to reduced AFC and that the mechanisms involved here should be investigated further given the potential therapeutic implications.

#### *Bacterial Infections and ENaC Activity*

A number of factors influence ENaC activity and expression in alveolar epithelial cells. ENaC expression is increased by glucocorticoids (26, 28, 64, 84) and decreased by interleukin-1 $\beta$  (128), interleukin-4 (41), and transforming growth factor- $\beta$  (38). The effects of tumor necrosis factor (TNF)- $\alpha$  are more controversial, with some studies showing that TNF $\alpha$  increases ENaC activity (40) and others showing that TNF $\alpha$  inhibits it. This controversy was reconciled by Braun et al. (10), who reported results indicating that the receptor-binding sites of TNF inhibit ENaC, whereas its lectin-like domain activates ENaC.

In rat alveolar epithelial cells, an inducer of cellular stress, cycloheximide, and inflammatory responses to lipopolysaccharide (LPS), a glycolipid from the outermost membrane of gram-negative bacteria, were recently shown to downregulate  $\alpha$ -ENaC mRNA levels (103). To understand the mechanisms involved, Migneault and colleagues (103) followed a time course of treatments and found that both methods decreased  $\alpha$ -ENaC by  $\sim 50\%$  in as little as 4 h. Interestingly, the effect of cycloheximide was not dependent on its ability to inhibit translation. Both treatments downregulated  $\alpha$ -ENaC message via the ERK and p38 MAPK pathways, although LPS appeared to inhibit  $\alpha$ -ENaC promoter activity, whereas cycloheximide inhibited ENaC through posttranscriptional effects. The authors posit that the LPS effects could be due to a proinflammatory response, whereas the cycloheximide response appeared to be due to a nonspecific stress response that did not involve reactive oxygen species (ROS) but apparently did affect proinflammatory cytokines (103). A difference between the two effectors was clear, since inhibition of either pathway was sufficient to block the cycloheximide effects, whereas simultaneous inhibition of both pathways was required to block



the LPS effect. Given that *Pseudomonas aeruginosa* is the most common bacterial infection in CF and that *P. aeruginosa* is known to downregulate ENaC in infected mouse lungs (27), this could suggest that any inflammatory or stress condition in the lungs would have a detrimental effect on ENaC activity, even without invoking reactive species, which are known to inhibit ENaC (32, 155).

#### Protein Kinase C and ENaC Activity

An important signaling molecule for regulating ENaC activity is protein kinase C (PKC). PKC activation inhibits ENaC activity (39, 88, 142), and, conversely, PKC inhibition enhances ENaC activity (88, 151). Although there is significant information about the role of PKC phosphorylation in the kidney (4, 39), there is only one isoform in the principal cells (69). There are, however, multiple isoforms of PKC in the lung (147), suggesting that there could be differences in PKC regulation in the lung. To test the role of PKC phosphorylation in the lung, Eaton et al. (35) used a mouse PKC $\alpha$  KO model. In cell-attached patch-clamp studies in AT2 cells, they demonstrated that the ENaC  $P_o$  and the number of channels were significantly decreased in the PKC $\alpha$  KO mice compared with controls. Using biochemical approaches, they demonstrated that all three ENaC subunits ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) were expressed at lower levels in the KO mouse lung and that this correlated with decreased Na<sup>+</sup> transport in AT2 cells (35). Because alveolar tissues are exposed to high levels of oxygen, they used a fluorescent superoxide reporter and lung slices to show that the PKC $\alpha$  KO animals produce nearly twofold more superoxide than the wild-type animals. The superoxide dismutase activities were also decreased in the cytosol and mitochondria in the KO animals. ERK1/2 activity was elevated in the KO lung (35), and ERK1/2 phosphorylation of ENaC promotes Nedd4-2 interactions with ENaC (7), which leads to ENaC ubiquitination and subsequent degradation. The ROS activates PKC $\delta$ , which reduces ERK1/2 dephosphorylation and leads to decreased amounts of ENaC. Eaton et al. also showed that the decreased ENaC  $P_o$  was attributed to PKC $\delta$  phosphorylation of myristoylated alanine-rich PKC substrate. Myristoylated alanine-rich PKC substrate (36), which acts as a scaffolding protein at the plasma membrane that promotes phosphatidylinositol phosphate interactions with ENaC and enhances activity, dissociates from the membrane after phosphorylation, which lowers the phosphatidylinositol phosphate concentration and the ENaC activity. To establish the role of ROS and PKC $\delta$  in this process, a ROS scavenger or PKC $\delta$  inhibitor was used in patch-clamp experiments, and these manipulations blocked these effects (35). From these data, Eaton et al. speculate that a PKC $\alpha$ -PKC $\delta$  double KO would cause ENaC activity to increase. There is evidence to indicate that reactive intermediates damage ENaC and inhibit Na<sup>+</sup>-driven AFC in vivo (18, 34, 56, 139). On the other hand, it has been shown that small amounts of superoxide are capable of upregulating ENaC and stimulating Na<sup>+</sup>-driven AFC in vivo (32, 47).

There is also evidence that another isoform of PKC (PKC $\zeta$ ) is involved in the downregulation of ENaC. Davis et al. (29) reported that ENaC was not activated by  $\beta_2$ -agonists in respiratory syncytial virus-infected mice, because KC (IL-8) promotes hydrolysis of GTP by the inhibitory  $\alpha$ -subunit of the receptor-associated heterotrimeric G protein (G $\alpha$ ), which then

activates PKC $\zeta$ . In turn, PKC $\zeta$  phosphorylates and activates G protein-coupled receptor kinase 2, which serine phosphorylates  $\beta_2$ -adrenergic receptor, uncoupling it from G $\alpha$ . Upon binding of  $\beta$ -agonist, these phosphorylated receptors are unable to activate adenylyl cyclase, and no AFC response is detected (29). Ji et al. (65) reported that severe acute respiratory syndrome coronavirus proteins decrease levels and activity of human ENaC via activation of PKC $\alpha/\beta$  and PKC $\zeta$ . Lazrak et al. (81) showed that activation of a number of PKC isoforms ( $\alpha$ ,  $\beta_1$ , and  $\zeta$ ) plays an essential role in the downregulation of ENaC by the M2 protein of influenza virus. Infection of lung epithelial cells with inactivated influenza virus A/PR/8/34 (PR8;H1N1) inhibited ENaC function and decreased Na<sup>+</sup> transport across airway murine distal epithelial cells, and these events were prevented by pretreatment with PKC inhibitors (20).

#### CF and a Mutation in the ENaC $\beta$ -Subunit

ASL levels and composition are regulated by a critical balance between Na<sup>+</sup> absorption and Cl<sup>-</sup>/bicarbonate secretion (23). In CF, the Cl<sup>-</sup> and bicarbonate channel CFTR has severely compromised surface expression and/or function, which leads to mucociliary transport defects, bacterial infections, inflammatory responses, and, eventually, lung failure. Furthermore, loss of CFTR function leads to dysregulated ENaC function (82). In an interesting study, Rauh et al. (122) examined a mutation in the  $\beta$ -subunit of ENaC ( $\beta$ V348M) that had been identified in a patient with severe CF-like symptoms (105). To fully characterize this mutation, they expressed the  $\alpha\beta\gamma$ -ENaC channels with wild-type ENaC or with the mutant  $\beta$ -subunit in *Xenopus laevis* oocytes and monitored whole cell and single-channel conductances. The analysis revealed that the  $P_o$  was increased nearly twofold with the  $\beta$ V348M mutation (122). This gain-of-function mutation was confirmed in transfected HEK 293 cells. Computational channel modeling, along with functional analysis, suggested that the gain-of-function open channel was caused by a destabilized closed-channel state. These studies are significant, since they clearly point to how elevation of ENaC activity in the presence of one mutant CF allele can promote a severe CF phenotype and support the idea that enhanced ENaC activity contributes to the pathophysiology of CF.

In that regard, it is interesting that an 18-amino acid peptide from residue G22–A39 of a natural inhibitor of ENaC in airway epithelial, short palate lung and nasal epithelial clone 1 (SPLUNC1) has been shown to be useful in treating Na<sup>+</sup> hyperabsorption in CF airway epithelial cultures (57). Treatment of CF has often involved correction of Cl<sup>-</sup> channel activity or suppression of ENaC activity, and this was further supported by studies of Lazrak and colleagues (83), who examined the role of inter- $\alpha$ -inhibitor (I $\alpha$ I), a protease inhibitor, on ENaC activity in CF. There are two pools of ENaC channels at the cell surface, an active proteolytically cleaved pool and an inactive noncleaved pool. The results show that I $\alpha$ I is present in the bronchoalveolar lavage fluid of children with CF (83), suggesting that it could potentially inhibit ENaC activity in CF patients and, thus, block Na<sup>+</sup> hyperabsorption. The results of Lazrak and colleagues, in lung slices from  $\Delta$ F508 CF mice, showed that I $\alpha$ I was an effective inhibitor of ENaC proteolysis and was able to decrease ENaC activity in

the lung epithelial cells from  $\Delta F508$  CF mice. Since reactive species activate PKC (32), these two signaling molecules may act in synergy to downregulate ENaC in a number of pathological conditions.

#### Other ENaC Subunits: $\delta$ -ENaC

The fourth subunit of ENaC, the  $\delta$ -subunit, was recently cloned in human and monkey (reviewed in Ref. 66). The  $\delta$ -subunit is expressed in epithelial and nonepithelial tissues and has an expression profile similar to the  $\gamma$ -subunit (66). High levels of expression of the  $\delta$ -subunit are found in a number of human tissues, including liver, heart, skeletal muscle, prostate, pituitary, smooth muscle, and lung. A prevailing view is that a functional ENaC is composed of at least one  $\alpha$ -like subunit ( $\alpha$  or  $\delta$ ) and that the  $\beta$ - and  $\gamma$ -subunits are required to amplify channel activity (66). In the lung, the  $\delta$ -ENaC subunit has been found in human primary alveolar cells and shown to contribute  $\sim 50\%$  of the amiloride-sensitive salt transport across human nasal epithelial cells (3). Interestingly, but unfortunately, the  $\delta$ -subunit is not expressed in the mouse (46), making functional analysis of this subunit more difficult. Highly selective  $\text{Na}^+$  channels have been reported to contain  $\alpha$ -,  $\beta$ -, and  $\gamma$ -ENaCs, whereas channels consisting of other combinations of subunits have decreased selectivity for  $\text{Na}^+$  over  $\text{K}^+$  and higher amiloride  $\text{IC}_{50}$  (18, 154). Furthermore, ENaC channels containing  $\delta$ -ENaC are activated by PKGII and cGMP, in addition to cAMP (106). Elevated levels of the  $\delta$ -subunit have been associated with chronic sinusitis and allergic disorders, and reduced expression has been associated with rhinovirus diseases (reviewed in Ref. 66). Children with the genetic deletion of this subunit are predisposed to respiratory infections and nasal congestion. Finally, whether channels containing  $\delta$ -ENaC are essential in lung fluid homeostasis will require in vivo and ex vivo studies in primates.

#### $\beta$ -Adrenergic Effects on Anion Secretion

ASL is regulated by  $\text{Na}^+$  channel absorption and  $\text{Cl}^-$  channel secretion, and Shamsuddin and Quinton (134) questioned the hypothesis that absorption and secretion have to occur in the same cells. In studies in Calu-3 cells, which are a model for serous cells of airway submucosal glands, Banga et al. (2) examined the role of epinephrine in cells that do not normally express ENaC. Interestingly, CFTR in Calu-3 cells is stimulated by nitric oxide and compounds that increase cGMP (17, 19). The results confirmed the lack of ENaC in these cells and showed that this  $\beta$ -adrenergic receptor agonist stimulated two  $\text{Cl}^-$  channels, CFTR and TMEM16A. TMEM16A was shown recently to be present in serous cells and Calu-3 cells (72), as well as human and mouse lung airway smooth muscle cells (42). Furthermore, isolated trachea from TMEM16A KO mice revealed reduced MCC (86), suggesting that both CFTR and TMEM16A are required for normal hydration in mice. Using inhibitors of CFTR and TMEM16A, Ousingsawat et al. (111) also found that the combination of inhibitors had a more profound effect than the additive effect of the separate inhibitors. This result supports the view that CFTR and TMEM16A are functionally linked, as proposed by Kunzelmann et al. (75), and that this linkage may involve  $\text{Ca}^{2+}$  elevations that may also stimulate CFTR (6). In addition, in human airway smooth muscle cells, activation of TMEM16A by intracellular  $\text{Ca}^{2+}$

secondary to oxidant stress results in membrane depolarization, which may contribute to increased airway reactivity (80).

#### Therapies: Stem Cells and Ion Transport

The epithelial lining of the lung must maintain a thin ASL for efficient gas exchange. Furthermore, fluid absorption out of the alveolar lumen requires active transport of  $\text{Na}^+$  from the apical surface of the pulmonary epithelium, across the apical and basolateral membranes, and into the interstitial space and/or bloodstream. Any factor that disrupts  $\text{Na}^+$  transport results in fluid accumulation and inefficient gas exchange. The importance of this transport process in the lung is clearly demonstrated in a number of human disease processes, where decreased  $\text{Na}^+$  absorption across the alveolar epithelium contributes to the pathophysiology of pulmonary diseases (reviewed in Refs. 94 and 98), including ALI and ARDS (54, 63, 98).

One potential therapy that holds a lot of promise is the use of bone marrow-derived mesenchymal stem cells (MSCs) because of their ability to differentiate into a number of cell types (51, 63, 117). For example, intravenous and intra-alveolar administrations of MSCs have been shown to decrease the severity of lung damage in a variety of models of ALI (51, 63, 73, 85). Interestingly, although earlier studies (110) reported increased engraftment of systemically administered MSCs in the alveolar epithelium after bleomycin lung injury, a number of other studies found protective effects of MSC therapy, despite low engraftment rates. Furthermore, the lung recovery rates were rapid (1–2 days), suggesting that the protective effects were not due to the MSCs themselves but, rather, their secreted products (51, 63, 73, 85).

With that idea in mind, Ionescu and colleagues (63) demonstrated that treatment with conditioned medium could improve ALI in mice. More recently, Goolaerts and colleagues (48) tested paracrine factors secreted by MSCs in an in vitro model of acute alveolar injury to determine the effectiveness of the treatments but, more importantly, to determine which factors were important for the therapeutic benefit. In this model, primary rat alveolar cells were exposed to hypoxia (3%  $\text{O}_2$ ) plus a mixture of cytokines present in ALI pulmonary edema (IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ ). Two types of conditioned media were used in these studies, MSC-conditioned medium from human MSCs exposed for 12 h to normoxia (MSC-M) and conditioned medium from MSCs exposed to hypoxia plus the cytokine mixture (HCYT-MSC-M). The HCYT-MSC-M used in this model was tested to mimic the effects of MSCs in the injured lung. The results from this study demonstrated that the inflammatory and hypoxic stress to the alveolar epithelial cells increased transepithelial permeability to albumin and decreased ENaC activity at the apical surface and that keratinocyte growth factor (KGF) secretion by MSCs, which was reduced in the HCYT-MSC-M samples, was required for recovery of AFC due to active  $\text{Na}^+$  reabsorption (48). These results support the idea that appropriate paracrine factors produced by MSCs in the injured lung hold therapeutic promise by restoring normal  $\text{Na}^+$  channel function and preventing alveolar flooding.

The role of KGF was supported by another study by McAuley and colleagues (101) in a human ex vivo lung model using lungs that were unsuitable for transplantation and had under-

gone a prolonged period of ischemia. They tested whether treatment of the lungs with human MSCs could restore normal AFC. Treatment with MSCs restored normal AFC but, more interestingly, KGF was required, since intrabronchial administration of a neutralizing antibody to KGF blocked AFC recovery. The authors suggest that these studies are significant, because they demonstrate that MSCs can enhance AFC in donor lungs with impaired AFC, and this is significant given that only 15–25% of donor lungs are transplanted (101, 116).

Influenza infection is known to damage ENaC and CFTR and decrease their activity in vitro and in vivo (20, 81, 91, 146, 148). Given the positive effects reported in other models of lung injury, Gotts and colleagues (50) investigated whether murine and human bone marrow-derived MSCs administered intravenously during the rapid phase of lung injury in influenza-infected mice would be beneficial in reducing epithelial injury. Although they did not directly measure AFC, their results indicate that administration of stem cells failed to improve lung water levels, bronchoalveolar lavage inflammation, or histology. One possible reason for this negative result comes from studies that show that swine bone marrow-derived MSCs express sialic acid receptors for hemagglutinin binding and, when infected by influenza, secrete proinflammatory cytokines, including TNF- $\alpha$  and IL-6 (68). The influenza-induced alveolar damage in the mouse lung peaked at 1 wk but appeared to last longer than 3 wk (50). These changes appeared to mimic those of ARDS, which highlights the profound effect of influenza infection on lung function.

#### *Inhibition of ENaC to Enhance ASL Hydration*

As mentioned above, hydration of the airways is regulated by active transport of Na<sup>+</sup> and Cl<sup>−</sup>, as well as bicarbonate transport, which maintains a ~7-mm PCL to facilitate cilia function and acts as a lubricant to keep the mucus layer away from the alveolar epithelial cell surface (1). This is a critical function, since hydration of the ASL influences the effectiveness of MCC and dehydration is believed to be a common feature of CF and COPD (1, 22, 23). Given that cigarette smoke decreases CFTR function (22, 95, 104, 121) and loss of CFTR elevates ENaC activity (15, 45, 58, 82, 90), Astrand and colleagues (1) tested the hypothesis that an ENaC inhibitor could be used to properly rehydrate airway cultures that were exposed to cigarette smoke and, thus, restore normal MCC. They tested the ability of a compound that was a potent ENaC inhibitor but, just as importantly, had a long half-life (>20 h) in rats (*compound A*) to reverse cigarette smoke-induced injury in lung epithelial cells. Their results demonstrate that pretreatment with *compound A* blocked cigarette smoke-induced ASL dehydration in the bronchial epithelial cultures and that the increased airway height correlated with increased MCC in vivo, supporting the view that ENaC inhibition is a viable approach for enhancing MCC and, therefore, a viable strategy for treatment of patients with chronic bronchitis. Although use of an ENaC inhibitor has been suggested for chronic bronchitis, the concern has always been that inhibitors such as amiloride were too short-acting to be effective in vivo, and importantly, any drug would have to be carefully tested to ensure that it had minimal effects on the kidney (107). In this case, *compound A* was used to demonstrate that inhibition of ENaC is a viable strategy in chronic lung diseases, but, unfor-

tunately, it would be unsuitable as a therapeutic because of its effects on renal handling of potassium (1).

#### *Summary and Conclusions*

Therapeutic strategies for curing or ameliorating lung pathologies such as acute lung disease, CF, and COPD require an understanding of the molecular mechanisms that control normal airway hydration and MCC and the factors that regulate these processes. One clear target is the ENaC in alveolar epithelial cells. Although significant progress is being made in developing long-acting ENaC inhibitors, finding one that does not have untoward effects in other tissues has proven to be difficult.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

#### AUTHOR CONTRIBUTIONS

S.M., R.B., and J.F.C. drafted the manuscript; S.M., R.B., and J.F.C. edited and revised the manuscript; S.M., R.B., and J.F.C. approved the final version of the manuscript.

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