Chapter 10

EPITHELIAL SODIUM CHANNELS IN THE ADULT LUNG – IMPORTANT MODULATORS OF PULMONARY HEALTH AND DISEASE

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Abstract: Absorption of excess fluid from the airways and alveolar lumen requires active vectorial transepithelial transport of sodium ions (Na^+) by alveolar type II and possibly type I cells. The rate-limiting step in this process is the activity of the heterotrimeric apical membrane epithelial Na^+ channel (ENaC). Pharmacologic inhibitors and genetic manipulations that disrupt Na^+ transport result in fluid accumulation within the lung and failure of gas exchange. The importance of Na^+ transport in the lung is also demonstrated in conditions such as ARDS, where abnormal absorption of Na^+ contributes to the pathophysiology of pulmonary disease. ENaC expression and function is influenced by diverse factors, such as oxygen tension, glucocorticoids, and cytoskeletal proteins. In addition, ENaC dysfunction has been shown to be induced by purinergic nucleotide activation of P2Y receptors (in paramyxoviral bronchiolitis) and reactive species (in acute lung injury). Finally, β-adrenergic agonists have been shown experimentally to reverse defects in ENaC function, and improve hypoxemia and pulmonary edema, and may provide a novel therapeutic modality for ARDS, although some viral lung pathogens appear to induce insensitivity to their actions.

Key Words: respiratory virus**,** β-adrenergic agonist**,** P2Y receptor**,** protein kinase C

THE ROLE OF SODIUM CHANNELS IN LUNG PHYSIOLOGY

For gas exchange to occur, the epithelium of the lung must maintain a humidified atmosphere with only a thin layer of fluid lining the airway surface. Absorption of fluid out of the airway and alveolar lumen requires active transport of sodium ions (Na^+) from the apical surface of the pulmonary epithelium, across the apical and basolateral membranes of epithelial cells, and into the interstitial space and/or bloodstream. Pharmacologic inhibitors and genetic manipulations that disrupt Na^+ transport result in fluid accumulation within the lung and failure of gas exchange. The importance of Na^+ transport in the lung is also demonstrated in several human disease processes, where

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abnormal absorption of Na^+ contributes to the pathophysiology of pulmonary disease.

While type I alveolar pneumocytes line >95% of the distal lung surface, alveolar type II pneumocytes (ATII cells) may mediate most of the ion and fluid transport (52). ATII cells, which make up 67% of the total number of alveolar epithelial cells, can be isolated with high purity, and grown as confluent monolayers (19; 51). Electrophysiological studies of cultured ATII cells have identified apical plasma membrane cation channels, referred to as epithelial Na^+ channels (ENaC; reviewed in (53)). These channels have a higher permeability to Na^+ than other cations and can be blocked by the diuretic drug amiloride (4). Na^+ ions diffuse passively into ATII cells (and possibly ATI cells, which have been shown to express ENaC subunit proteins and to transport Na^+ ions in vitro (7; 38)) through these apical cation channels (36; 76; 76) and are extruded across the basolateral membranes by the ouabain-sensitive Na^+,K^+-ATPase (20). While the driving force for Na^+ transport is produced by the basolateral Na^+,K^+-ATPase, it is the apical entry of Na^+ ions through ENaC which is the rate-limiting step for Na^+ flux. Indeed, the apical plasma membrane ENaC channels offer more than 90% of the overall resistance to transepithelial Na^+ transport.

The expression and function of ENaC is highly regulated. Multiple hormones and signaling pathways influence not only expression of the channels, but also post-translational modifications that regulate channel function. By understanding Na^+ transport at the molecular level, we can better understand the molecular pathogenesis of lung disease and design more appropriate therapies and interventions.

Biology of ENaC in the lung

ENaC is a heterotrimer of 3 transmembrane subunits (α, β, and γ), which are expressed in unequal proportions in respiratory epithelia (12), although the exact stoichiometry remains controversial. Some studies have indicated that ENaC forms a tetrameric complex (2α, β, γ) (21), while others have provided data indicating that the ENaC channel is a much larger complex (3α, 3β, 3γ) (68). mRNA for all 3 subunits of ENaC is present in the lungs of both humans and mice (10), but the Na^+ channels identified to date in airway epithelia display variable biophysical characteristics (single-channel conductance, P_{Na}/P_k, and affinity for amiloride and its ethylisopropyl analog (53)). For example, expression of α, β, and γ ENaC in Xenopus oocytes is associated with formation of highly Na^+ selective cation channels (12), but in ATII cells both nonselective and highly Na^+ selective cation channels have been identified (76). It appears that αENaC is sufficient to form functional amiloride-sensitive NSC channels, but the presence of β and γ subunits significantly enhances channel activity and substantively changes gating characteristics of the channel to the HSC form (12; 34; 36).

ENaC channels are constitutively open at the plasma membrane and do not appear to require additional activation (12). However, factors that influence both ENaC mRNA and protein levels can potentially modulate amiloride-sensitive Na^+ transport in the lung. Second messengers and signaling molecules may be able to alter the open probability of ENaC either by direct modification (phosphorylation/dephosphorylation) or by altering protein-protein interactions. While ENaC expression and function is known to be influenced by diverse factors, such as oxygen tension (62), glucocorticoids (73), and cytoskeletal proteins (5; 64), this review will concentrate upon three systems which

we have found to be involved in the pathogenesis of ENaC dysfunction in adult lung disease: the purinergic nucleotide system, reactive species, and β-adrenergic/cAMP agonists.

ENaC in adult lung disease

Na^+ transport appears to be essential for maintenance of a normal gas diffusion distance in the adult lung. In adults with acute respiratory distress syndrome (ARDS), Matthay and Wiener-Kronish (54) found a positive correlation between the ability of the alveolar epithelium to transport Na^+ actively and the rate of resolution of noncardiogenic pulmonary edema. Similarly, instillation of the epithelial Na^+ channel blocker phenamil into the lungs of rats exposed to hyperoxia resulted in higher levels of extravascular lung fluid volumes 24 hours later (75). Interestingly, the venom of a South American scorpion (Tityus serrulatus), which causes fatal respiratory failure and pulmonary edema, also decreases lung liquid clearance, probably by downregulating Na^+,K^+-ATPase in the alveolar epithelium (13). Finally, patients with systemic pseudohypoaldosteronism, caused by loss of function mutations in the genes encoding ENaC subunits, completely lack electrogenic Na^+ transport in the upper and lower airways. In some cases, pseudohypoaldosteronism results in a doubling of ALF volume, persistent rhinorrhea, and recurrent respiratory illness (39).

Effect of pulmonary pathogens on Na^+ transport

Despite the fact that fluid and mucus accumulation in airways and lung tissue is a major component of most respiratory infections (48), the effect of pathogens on respiratory epithelial Na^+ transport has not been studied in detail. Several lung pathogens have been shown to inhibit Na^+ transport by respiratory epithelia *in vitro*. Mycoplasma pulmonis inhibits amiloride-sensitive Na^+ absorption and cholinergic-stimulated Cl^- secretion by C57BL/6 mouse tracheal epithelial cells (45). Similarly, Pseudomonas aeruginosa rhamnolipids inhibit amiloride-sensitive Na^+ transport by ovine tracheal epithelium (26), while the hemolysin blocks active Na^+ uptake and Cl^- secretion by canine bronchial epithelium (71). Mycobacterium tuberculosis (77), pneumotropic, but not neurotropic, influenza A virus (42), and Sendai virus (43) have also been shown to inhibit ENaC activity in vitro. Influenza A virus rapidly (within 60 minutes of infection) inhibits amiloride-sensitive Na^+ transport by mouse tracheal epithelial cells. This inhibition is mediated by binding of viral hemagglutinin to cell surface sialic acid moieties, and subsequent activation of phospholipase C and PKC.

Interestingly, the inhibitory effects of pathogens on ENaC found in vitro have not always been found in vivo. For example, in rats with P. aeruginosa pneumonia, alveolar fluid clearance (AFC), which is a functional index of ENaC function, increased 24 hours after infection, and this increase, which was inhibited by amiloride, was at least partially mediated by TNF-α (63). Similarly, instillation of Escherichia coli endotoxin into the lungs of rats resulted in a significant increase in AFC at 24 and 40 hours (25). Whether such increases in AFC have detrimental pathophysiologic consequences, or whether they are the result of sublethal injury to the alveolar epithelium resulting in its repopulation with increased numbers of ATII cells, remains to be determined. Nev-

ertheless, some pulmonary pathogens may in contrast induce hypoxemia as a result of inhibition of AFC. For example, we recently reported that replicating respiratory syncytial virus (RSV) reduces the AFC of the bronchoalveolar epithelium in vivo (Fig 1), without inducing detectable epithelial cell death or an increase in alveolar permeability to albumin (17). Interestingly, we found that RSV-mediated inhibition of AFC was not related to viral loads per se: instead it was mediated by uridine triphosphate (UTP), acting in autocrine fashion on P2YR on bronchoalveolar epithelial cells. Specifically, we found increased levels of UTP in the bronchoalveolar lavage fluid of Balb/c mice 2 days following RSV infection (Fig 2). Moreover, reduced AFC was associated with increased lung water content, and peripheral hypoxemia (16). Addition of apyrase, which degrades both ATP and UTP, or XAMR0721 (a P2Y inhibitor) in the instillate prevented the decrease of AFC (17). Finally, UTP instilled in the alveolar space of Balb/c mice decreased AFC (Fig 3). Reduced AFC may result in formation of an increased volume of fluid mucus, airway congestion, and rhinorrhea, all features of severe RSV disease.

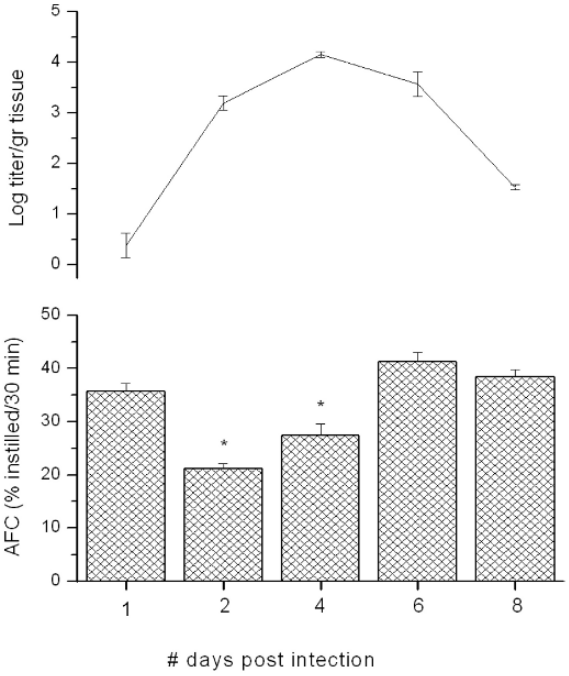

Figure 1. Intranasal infection of BALB/c mice with RSV results in significant inhibition of alveolar fluid clearance (AFC) at days 2 and 4 after infection. Mice were infectected with $2x10^6$ plaque forming units of RSV, suspended in 100 µl of buffer, intranasally as previously described (17). Alveolar fluid clearance (lower pannel; expressed as % of instilled fluid per 30 min) was measured across anesthetized, ventilated mice, with normal oxygenation and acid-base balance. n = 6-23 per group, as previously described (17). Mock infection had no effect on AFC. AFC was inhibited by 43% at day 2 and by 26% at day 4. RSV titers in lung tissues (upper pannel; expressed as the log of RSV PFUs per gr lung tissue) peaked at 4 days post infection and then decreased. Threshold of detection is 1 log. Notice the lack of correlation between RSV titers and AFC. Values are means ± SE. *$p<0.005$, compared with uninfected mice. Modified from ref 17.

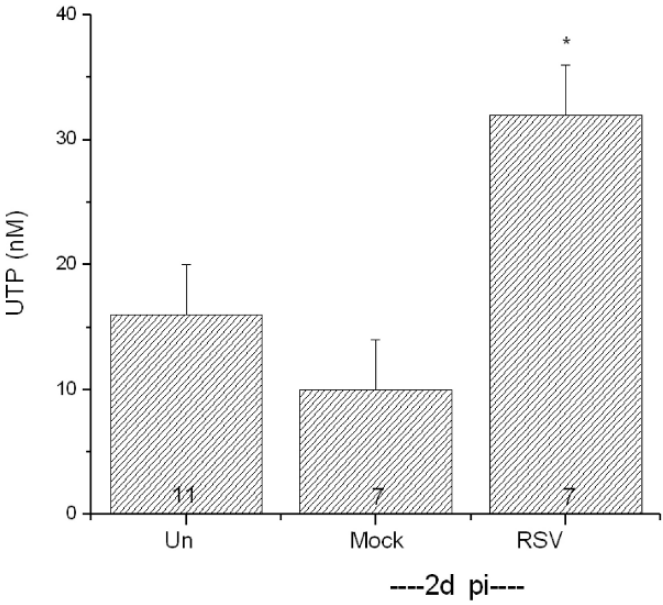

Figure. 2. RSV infection increases UTP levels in the BAL of Balb/c mice. Mice were infected with RSV as described in the legend of Figure 1. After 2 days, they were sacrificed and their lungs were lavaged. Endogenous nucleotidases in BAL fluid were heat denatured (100°C, 3 minutes) and UTP content was measured using the UDP-glucose pyrophosphorylase as previously described (16). Numbers are means ±1 SEM; * $<p<0.005$ compared to either unistilled or mock instilled mice. Labels as follows: Un = Uninstilled mice; Mock = Mock infected mice; RSV = infected with 2 x 10^6 plaque forming units of RSV. Numbers in bars indicate numbers of mice in each group.

ENaC modulation by purinergic nucleotides

Purinergic 5'-nucleotides are known modulators of ENaC activity in respiratory epithelial cells. Relatively large amounts of adenosine triphosphate (ATP) and UTP are released by human respiratory epithelial cells in vitro, although the underlying pathway for nucleotide release remains undefined (30; 46). Released ATP is rapidly metabolized to a mixture of ADP, AMP, and adenosine. ATP, its metabolites, and UTP each have inhibitory effects on ENaC, mediated via interaction with purinergic receptors expressed on respiratory epithelial cells (11; 40). Both ATP and UTP are known to inhibit respiratory epithelial Na^+ absorption in vitro (32; 33; 35; 50), via interaction with P2Y purinoceptors (P2YR). In vivo, UTP administered at pharmacologic doses (100 μM) to human subjects has also been shown to induce Cl^- secretion by nasal epithelium (41) and, when given by aerosol, to promote mucociliary clearance, although, interestingly, in the presence of amiloride, it also induced mild hypoxemia (60).

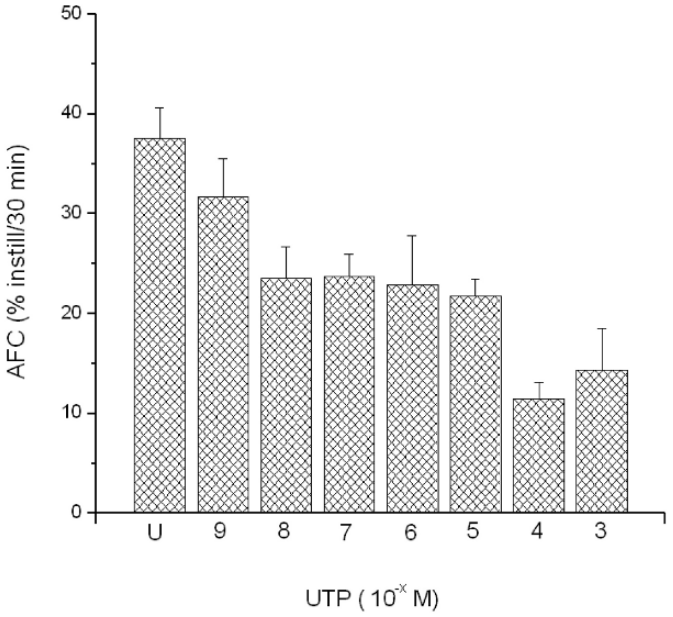

Figure 3. Instillation of UTP in the alveolar space of Balb/c mice decreases AFC. To confirm that UTP alone can recapitulate the inhibitory effects of RSV on alveolar fluid clearance, we instilled 5% BSA (the standard instillate for the measurement of AFC) containing ten-fold dilutions of UTP into the lungs of normal mice and determined AFC 30 min later. Shown values are means ± 1 SEM (n 3-4 per group). Final doses of UTP (from 1mM to 10 nM) had a significant inhibitory effect on AFC, but 1 nM UTP had no effect. 1 mM and 100 μM UTP induced significantly greater inhibition of AFC than that caused by RSV (62% and 70%, respectively), while doses from 1 μM to 10 nM caused inhibition similar to that induced by infection with RSV for 2 or 4 days (42-36%). Modified from ref. 17.

Protein kinase C as a central regulator of ENaC function?

Until recently, the mechanism by which nucleotide binding to P2YR might induce reduced ENaC activity has remained unclear. While downstream signaling events mediating ENaC downregulation have not yet been fully defined, it is known that P2YR are G-protein-coupled, and act via the inositol phosphate pathway to stimulate calcium release from intracellular stores, but can also act via multiple secondary signal transduction pathways including protein kinase C (PKC) (11). Activation of PKC has been shown to reduce ENaC activity and modify its subunit composition, although the isoforms of PKC involved have not been defined. Inhibition of PKC rapidly increased P_o and appearance of new channels in patches of A6 cells (47). In contrast, stimulation of PKC inhibited whole-cell currents in Xenopus oocytes (2). Likewise, PKC activation decreased expression of both β and γ, but not α ENaC subunit proteins in A6 cells by 3h and 14h, respectively, and also resulted in a decrease in transepithelial Na^+ reabsorption (70).

Recent data indicates that P2YR-mediated ENaC downregulation may also involve activation of the ubiquitin-proteasome pathway, which is an important regulator of ENaC function. The half-life of ENaC in mammalian cell membranes is short (less than 1 hour). ENaC is ubiquitinated in vivo on the α and γ, but not β subunits (69). Inhibition of ubiquitination or the proteasome results in increased channel activity, due to an increase in the number of channels present at the plasma membrane (49). Ubiquitination (ATP-dependent serial addition of ubiquitin monomers to lysine residues on proteins), which targets proteins for rapid degradation by the proteasome, is catalyzed by the sequential action of ubiquitin-activating, ubiquitin-conjugating, and ubiquitin protein ligase enzymes (29). Neural precursor cell-expressed developmentally downregulated protein 4 (Nedd4) is the ubiquitin-protein ligase required for ubiquitination of ENaC (69). Nedd4 directly regulates basal ENaC activity by modulating channel stability at the cell surface. In the lung, Nedd4 is mainly expressed in the epithelia lining the airways and in the distal respiratory epithelium, a pattern of expression similar to that of ENaC (8). Interestingly, the interaction between ENaC and Nedd4 is disrupted in Liddle Syndrome, a hereditable form of salt-sensitive hypertension (1). Liddle syndrome mutations in the βENaC cytoplasmic domain disrupt the association of Nedd4 with the C-terminus of βENaC. As a result, ENaC has a longer half-life in the plasma membrane and is less efficiently internalized and degraded. This leads to increased amiloride-sensitive current at the apical membrane and increased salt absorption.

The link between PKC and the ubiquitin-proteasome pathway has just recently been made clear. In A6 cells, PKC has been shown to activate the mitogen-activated protein (MAP) kinase Raf-1, and the MAP kinase kinases MAPK/ERK (MEK) 1 and 2. Activation of MEK 1 and 2 was shown to enhance phosphorylation of β and γ, but not α ENaC (67). This phosphorylation event facilitates binding of Nedd4-2 to ENaC, which may then promote ENaC internalization and removal from the cell surface (69). Therefore, purinergic stimulation and PKC activation may decrease ENaC function both through altered ENaC phosphorylation and altered ENaC degradation.

Inflammatory mediators of ENaC dysfunction in pulmonary disease

Reactive oxygen/nitrogen species (RONS), such as the free radicals nitric oxide (•NO) and nitric dioxide ($•NO_2$) as well as peroxynitrite anions ($ONOO^-$), are known to inhibit the activity of both ENaC (31) and the ATII cell Na^+/K^+ ATPase (72) in vitro, via both cGMP-dependent and cGMP-independent mechanisms. In pulmonary inflammatory disease, increased levels of RONS may directly modify ion transporters, disrupt their association with chaperone or structural proteins (such as actin), or alter signal transduction pathways, all of which may result in impaired Na^+ absorption across the alveolar epithelium. Nitrotyrosine (the stable by-product of $•NO_2$ reaction with tyrosine residues in proteins (3)) has been detected in the lungs of patients with acute lung injury (44) and those with hantavirus cardiopulmonary syndrome (HCPS) (18). Likewise, both nitrotyrosine and large amounts of nitrate, the stable by-product of peroxynitrite and nitrogen dioxide, have been found in the lavage fluids of patients with acute lung injury and the plasma of hantavirus cardiopulmonary syndrome cases (18). These find-

ings indicate that reactive oxygen-nitrogen species are produced in the lungs of patients with inflammatory disease, and may contribute to its pathogenesis. In a recent study, Modelska et al. (56) showed that absorption of isotonic fluid, secondary to Na^+ absorption across the alveolar space, was inhibited followed prolonged hemorrhagic shock. Moreover, instillation of aminoguanidine, a nitric oxide synthase inhibitor, restored fluid absorption to normal levels. Thus, increased production of reactive oxygen-nitrogen species by lung epithelial or inflammatory cells may modify molecules required for Na^+ transport across the alveolar epithelium.

Finally, it should also be noted that certain proinflammatory cytokines have also been shown to directly alter Na^+ transport by respiratory epithelial cells in vitro. Specifically, Na^+ transport is inhibited by IFN-γ (23), and IL-4 (24), while TNF-α has been shown to both increase (22; 63) and reduce (15) Na^+ transport, in vivo.

Effect of β-adrenergic agonists on ENaC function

β-adrenergic receptor agonists (β-agonists) have been shown to improve AFC in animal models of lung injury in which AFC is impaired, by increasing the activity of both epithelial Na^+ channels and Na^+, K^+ ATPase (reviewed in (58)). β-agonist prophylaxis has also been shown to be of value in reducing the incidence of high altitude pulmonary edema (itself a consequence of impaired AFC secondary to hypoxia at high altitude) in susceptible mountaineers (66), and intravenous salbutamol treatment can reduce extravascular lung water in patients with acute lung injury (61). Because of such encouraging findings, the National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Network is planning to conduct a large, multicenter, prospective clinical trial to test the potential efficacy of the aerosolized β-agonist albuterol in ventilated patients with ARDS (74).

Stimulatory effects of β-agonists on AFC have been shown to be mediated via activation of adenylyl cyclase, which generates cAMP and thereby stimulates cAMP-dependent protein kinase production (protein kinase A; reviewed in (37)). PKA phosphorylates cytoskeletal proteins and promotes both exocytosis to the cell membrane and direct phosphorylation of ENaC β and γ subunits (5). β-agonists also increase the expression of ENaC α-subunit mRNA and protein (55), phosphorylation-dependent translocation of Na^+, K^+ ATPase pumps from intracellular pools to the basolateral membrane of epithelial cells (6), and apical Cl^- flux through the cystic fibrosis transmembrane conductance regulator (CFTR) (59). However, while it appears that functional β-AR are essential for adaptation to pulmonary edema, it remains unclear whether they are required for maintenance of alveolar fluid balance in the normal lung (58).

Interestingly, several respiratory tract viral pathogens have been shown to modulate β-adrenergic receptor agonist function. Tracheal smooth muscle from influenza virus-infected mice has reduced sensitivity to β-agonists and forskolin ex vivo (28), and airway segments taken from ovalbumin-sensitized guinea pigs exhibit impaired bronchodilator responses following infection with parainfluenza 3 virus (9). Likewise, human airway smooth muscle tissue exhibits reduced responsiveness to β-agonists, associated with increased $G\alpha_i$ expression, following in vitro infection with rhinovirus (27) and RSV (57). Finally, we have recently found that RSV induces heterologous desensitiza-

tion of bronchoalveolar epithelial β-AR in vivo (Ian Davis, unpublished observations). These findings have important implications for dysregulation of other β-agonist-mediated responses in respiratory epithelium and airway smooth muscle following viral infection: β-agonists are known to promote mucociliary clearance and fluid secretion by submucosal glands, may have anti-inflammatory effects, and are widely used as bronchodilators (reviewed in (65)).

CONCLUSIONS

Na^+ transport in the distal lung is required for normal lung function. Defective Na^+ absorption may contribute to the pathogenesis of acute and chronic lung disease. As the molecular mechanisms of lung injury and disease are better characterized, we may better understand the contribution of ENaC function to pulmonary disease. The demonstration that RONS released by activated alveolar macrophages down regulate the activity of alveolar epithelial cell Na^+ channels (14) provides an example of how mediators of lung injury could influence Na^+ absorption during the progression of pneumonia or ARDS. Manipulating ENaC function may provide new treatments for both ARDS and pulmonary infectious diseases. For example, strategies that inhibit UTP-P2YR interaction can now be evaluated as therapeutics for RSV pneumonitis, a condition for which specific antiviral drugs are sadly lacking. Identification of additional targets for regulating ENaC expression and function in the lung may provide further opportunities for clinicians to target Na^+ absorption in the lung. Moreover, by studying Na^+ channel physiology in the context of human lung disease, we may learn more about the basic physiology of distal lung transport.

ABBREVIATIONS

AFC = alveolar fluid clearance; ATII = alveolar type II cells; ENaC = epithelial sodium channels; RSV = respiratory syncytial virus; UTP = urine triphosphate; RONS = reactive oxygen-nitrogen

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