The Role of Hydrocortisone on the Development and Maintenance of Ion Transport Pathways Essential to Mucociliary Clearance in Differentiated Normal Human Bronchial Epithelial Cells

A DISSERTATION
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Nathan A. Zaidman

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNDER THE MENTORSHIP OF
Scott M. O'Grady, PhD
Angela Panoskaltsis-Mortari, PhD

September 2016

Acknowledgements

The present thesis is the culmination of five years of research that would not have been possible without the significant contributions of my mentor and advisor, Dr. Scott M. O'Grady. Professor O'Grady provided me the independence to grow, the challenge to learn and the support to succeed. I am eternally grateful for his collaboration, his care, and most importantly, his criticism. My accomplishments, now and in the future, are merely a reflection of his mentorship.

I would also like to acknowledge the faculties of the Integrative Biology & Physiology and Animal Science departments. They provided me with exceptional community and respect throughout my graduate work. Their willingness not only to entertain my questions, but more importantly to question my questions have made me a far better student and helped me to understand the paths ahead of me. I would like to especially thank Professors John Osborn, Alessandro Bartolomucci, and DeWayne Townsend from IBP and Professors Milena Saqui-Salces, Laura Mauro and Chris Faulk from Animal Science. Furthermore, the staffs of both departments have been nothing short of spectacular during my tenure. Notably, the contributions of Jane Barnard, Kim Reno, Jacqueline Lee, Randi Lundell, Bill Klein, and Crystal Langeberg.

I need to thank the other members of Dr. O'Grady's laboratory who assisted me with my work throughout my graduate studies. Thank you to Peter Maniak for helping me every single day, Elizabeth Peitzman, Nanda Patil, Yotesawee Srisomboon, Tamene Melkamu and Francesca Milavetz. Additionally, I would like to acknowledge the staff of the Mortari laboratory, specifically Carolyn Meyer and Andy Price, for being great colleagues and friends.

I would like to thank my peers in the Integrative Biology & Physiology PhD program for their encouragement and enthusiasm. Specifically, I would like to acknowledge Jason Foss for proving it could be done, Cheryl Cero for her persistence and company, Anthony Vetter for his attention, and Tim Matsuura for his friendship and acuity. Moreover, the conversations and relationships I have had and kept with all of my peers has been the overarching theme of my graduate studies.

Additionally, I would like to thank the members of my thesis committee, Professors Angela Panoskaltsis-Mortari, Al Beitz and John Osborn. I am grateful for their insight, their instruction and, most importantly, their approval.

I was fortunate to be awarded a pre-doctoral research fellowship from the National Institute of Biomedical Imaging and Bioengineering, which supported me financially during two years of my PhD. I am thankful to all of the individuals at the NIH who reviewed my submission and assisted me throughout the duration of the award.

I would be remiss to not thank and acknowledge the work of the building maintenance and janitorial staff in Haecker Hall. Without their support I surely would not have completed my thesis. I am exceptionally grateful for their contributions and labor.

Finally, I would like to acknowledge my friends and family for their curiosity and companionship. I need to thank my parents, Enrica and Brian, for great tolerance and encouragement. I also would like to thank my friends for their loyalty and compassion. Big ups to Snake, Squid, Steve, Pete and Caitlyn, Matthew P, Mike and Alyssa, Jack and Nicki, Rusty, Joe, Tones, Haley, Chuck and Karls. Most of all, I need to thank my dear friend Sarah for her love and enduring support.

Dedication

This thesis is dedicated to Darko, Alessandro, Michelina, Antonio, Luciene, Abhijit, Lynn, Dina, Aline, Elisabeth and Victor, for helping me achieve my goals.

Abstract

The airway epithelium establishes a boundary between the internal and the external environment that protects against potential injury and infection caused by inhaled particles, debris and microbial pathogens. In order to maintain the health of the lungs and surrounding tissues, the epithelium utilizes numerous defense mechanisms that complement the barrier function of the epithelium. These include the expression of a broad array of innate immune receptors including Toll-like, NOD-like and RIG-I receptors which facilitate the expression and secretion of multiple defense molecules, cytokines and chemokines essential for recruitment and activation of immune cells. Chronic inflammation of the airways due to prolonged or repeated exposure to noxious agents can result in epithelial remodeling and fibrosis that leaves the airways more susceptible to infection due to disruption of innate immune processes. Loss of epithelial integrity compromises normal mucosal defense, underscoring the important relationship between structure and function of the airway epithelium.

The pseudostratified airway epithelium is composed of multiple cell types. Multipotent, progenitor basal cells are transit-amplifying cells that reside along the basal lamina of the airway which differentiate into surface cells. Basal cells are instrumental in the maintenance of normal epithelial structure and function as well as driving orderly regeneration after injury. Differentiated surface cells can be divided into two distinct lineages: secretory and ciliated. Secretory cells synthesize and secrete gel-forming polymeric mucins that absorb water to form mucus. Ciliated cells propel the mucus gel towards the pharynx and out of the lungs by the directional, synchronized beating of cilia on the cell surface, effectively removing entrapped particles and pathogens from the lungs by a process known as mucociliary clearance.

Current treatment strategies for patients with asthma, COPD and other inflammatory airway disorders emphasize a combined therapy involving inhaled corticosteroid (ICS) and long-acting β_2-agonists (LABA) aimed at reducing inflammation and increasing airway caliber. LABAs, such as Salmeterol and Formoterol, are effective long-acting inhaled bronchodilators. These drugs stimulate increases in cAMP which

activates protein kinase A (PKA) dependent signaling pathways in airway smooth muscle, leading to relaxation and a decrease in airway resistance. The therapeutic benefit of ICS treatment is attributed to transrepression of specific genes induced by proinflammatory transcription factors such as NF-kB and AP-1, limiting airway hyperresponsiveness. Other effects of corticosteroids on airway epithelial cells suggest a role in surface cell diversity by increasing the number of ciliated epithelial cells and decreasing mucus hypersecretion, therefore promoting mucociliary clearance.

Although effective at reducing inflammatory immune reactions and airway hyperresponsiveness, the effects of combined therapy on the airway epithelium are not as well characterized. Therefore, the overall goal of the present thesis was to investigate the role of glucocorticoids in the development of the ion transport phenotype of bronchial epithelial cells during differentiation. We hypothesized that hydrocortisone (HC) is necessary for in vitro differentiation of airway basal cells into a pseudostratified epithelium with ciliated and secretory surface cells. Additionally, we hypothesized a role for HC in the development of normal transepithelial ion-transport pathways, both transcellular and paracellular, that are essential for mucociliary clearance. The results of the present studies demonstrate that early glucocorticoid exposure affects the development and maintenance of specific ion-transport pathways required for mucociliary clearance. These findings indicate that ICS treatment supports mucosal defense through direct interactions with the airway epithelium.

Table of Contents

List of Figures ..... **viii**

Chapter 1: Background, Objective and Specific Aims **1**

Background 2

Pulmonary Epithelium Anatomy and Composition 2

Airway Innate Immune Function 3

Asthma 5

Mucociliary Clearance 8

Transcellular Sodium Absorption 10

Transcellular Chloride Secretion 12

Potassium Channels Involved in MCC 14

Important Signaling Pathways in MCC 15

Transcellular Water Transport 17

Paracellular Pathway 17

Electrophysiologic Measurements 20

In Vitro Differentiation of Normal Human Bronchial Epithelial Cells 22

Objective and Specific Aims 27

Chapter 2: Differentiation of Human Bronchial Epithelial Cells: Role of Hydrocortisone in Development of Ion Transport Pathways Involved in Mucociliary Clearance **29**

Synopsis 30

Introduction 31

Materials and Methods 33

Results 36

NHBE cells differentiate into a ciliated-pseudostratified epithelium 36

Hydrocortisone-sensitive sodium and anion transport in differentiated NHBE cells 37

Anion secretion increases following β2-AR stimulation 39

Discussion 40

Summary and Conclusions 44

Chapter 3: Large Conductance Ca^{2+}-activated K^+ Channel Activation by Purinergic Receptor Agonists Requires Hydrocortisone in Differentiated Normal Human Bronchial Epithelial Cells.....59

Synopsis 60

Introduction 61

Materials and Methods 63

Results 65

Paxilline-sensitive I_{SC} is HC dependent in differentiated NHBE cells.....65

NHBE Control and HC0 cells express BK subunits 66

Differentiated NHBE cells have distinct P2YR signaling domains 67

The BK activator NS11021 potentiates the effect of UTP 68

PKC activity is required for UTP stimulated BK activity.....68

Hydrocortisone reduces STREX-variant KCNMA1 transcription.....69

Differentiated NHBE express CaCCs as well as ClC chloride channels 70

Discussion 70

Summary and Conclusions.....75

Chapter 4: Regulation of Claudin Expression and Paracellular Ion Transport by Glucocorticoids in Differentiated Human Bronchial Epithelial Cells 91

Synopsis 92

Introduction 93

Materials and Methods 95

Results 97

Discussion 99

Chapter 5: Summary, Conclusions, Perspectives and Future Directions.....111

Summary and Conclusions.....112

Perspectives and Future Studies.....115

References.....121

List of Figures

Chapter 2: Differentiation of Human Bronchial Epithelial Cells: Role of Hydrocortisone in Development of Ion Transport Pathways Involved in Mucociliary Clearance

Figure 2.1 NHBE cells develop tight junctions and form electrically tight monolayers during differentiation 46-47

Figure 2.2 Differentiated NHBE cells express mRNAs associated with bronchial basal cells and surface cells 48-49

Figure 2.3 Immunocytochemistry of differentiated NHBE cells reveals a pseudostratified epithelium with ciliated and mucin-containing cells 50

Figure 2.4 Day 8 HC0 cells have reduced benzamil-sensitive current but normal $CFTR_{inh}$-172-sensitive current compared to hydrocortisone treated controls 51

Figure 2.5 Day 24 HC0 and HC8 cells have reduced total and benzamil-sensitive currents compared to control 52

Figure 2.6 HC0 cells express lower ENaC mRNA levels and have less apically localized ENaCα subunits compared to control and HC8 cells 53-54

Figure 2.7 Differentiated NHBE monolayers display increased Isc responses after apical treatment with the selective β2-AR agonist salbutamol 55-56

Figure 2.8 CFTR and β2-AR co-localize at the apical membrane of differentiated NHBE monolayers 57-58

Chapter 3: Large Conductance Ca^{2+}-activated K^+ Channel Activation by Purinergic Receptor Agonists Requires Hydrocortisone in Differentiated Normal Human Bronchial Epithelial Cells

Figure 3.1 NHBE cells exhibit paxilline-sensitive K^+ secretion after stimulation with UTP that was not observed in HC0 cells..... 77-78

Figure 3.2 Differentiated NHBE cells express the α_1, β_3, β_4 and LRRC26 γ subunits of BK ($K_{Ca}1.1$) potassium channels..... 79-80

Figure 3.3 NHBE cells express $P2Y_4$ receptors in the apical membrane, forming a pool of P2YR that are not stimulated by ATP-γ-S 81-82

Figure 3.4 The small molecule BK activator NS11021 potentiates the stimulatory effect of UTP by increasing the duration of the I_{SC} response..... 83-84

Figure 3.5 PKC inhibition blocks BK activation by UTP..... 85-86

Figure 3.6 Differentiated NHBE cells express DIDS-sensitive ClC-Ka/b chloride channels as well as the calcium-activated chloride channel ANO1 (TMEM16A) .. 87-88

Figure 3.7 Cell model showing the effects of ATP/UTP on specific ion transport processes in control and HC0 epithelia 89-90

Chapter 4: Differentiation of Human Bronchial Epithelial Cells: Role of Hydrocortisone in Development of Ion Transport Pathways Involved in Mucociliary Clearance

Figure 4.1 NHBE cells exhibit dynamic regulation of claudin mRNA expression during mucociliary differentiation 103-104

Figure 4.2 Characterization of paracellular Na+ and Cl- permeability in control and HC0 NHBE epithelia 105

Figure 4.3 Claudins localize at sites outside of tight junctions in differentiated NHBE cells 106

Figure 4.4 Immunocytochemistry demonstrating claudin-3, -4 and claudin-10 localization in in differentiated NHBE cells 107

Figure 4.5 Claudin-3 localizes in the cilia of differentiated NHBE surface cells 108

Figure 4.6 Claudin-3, -8 and -9, as well as P2Y2 receptors, co-localize in the cilia of differentiated NHBE surface cells 109-110

Chapter 1

Background, Objective and Specific Aims

Background

Pulmonary Epithelium Anatomy and Composition

The human airways are composed of a branched network of ducts that allow for bidirectional transfer of approximately six liters of air per minute between the internal and external environments (288). The largest airway, the trachea, has an internal diameter of about 12mm in humans and is supported by cartilage rings that extend for several bronchial generations into the lung (296). There are an estimated 54,000 total branches beginning at the trachea and terminating with the alveoli; the first 16 generations are considered the conducting zone while the final seven are known as the respiratory zone (288). Submucosal glands, sites of significant mucus and water secretion (105), extend over much of the conducting zone and exist between the mucosal surface of the airways and the supporting cartilage or smooth muscle. Terminal bronchioles of the respiratory zone lead to alveoli and alveolar sacs. There are on average 500 million alveoli in the human lung, each covered extensively by a network of capillaries that facilitate gas exchange (261).

The airways are lined by an epithelium with a cellular composition and structure that varies along the proximal-distal axis to meet local functional requirements (288). The epithelium of the upper airways is described as pseudostratified and contains multiple cells types. Basal cells, named for their proximity to the basal lamina, are common to epithelia throughout the body (296). Airway basal cells are multipotent progenitor cells that initiate both normal recurrent cell replacement within the epithelium as well as the orderly regeneration of epithelial tissue after injury (296). They are characterized by the expression of specific markers (cytokeratins, Trp63, NGFR) and constitute about 30% of the total epithelial population of the large, conducting airways (8, 250, 295).

Basal cell differentiation produces surface cells which can be broadly classified as either ciliated or secretory, each representing about one third of the total epithelium. The remainder of the cellular population includes neuroendocrine cells, dendritic cells and less characterized ‘intermediate’ cells. Basal cell differentiation is regulated by multiple signaling pathways including FGF, EGF, WNT, retinoic acid (RA), and Notch (244, 288,

397). Normal human bronchial epithelial cells (NHBE) cultured in the absence of RA generate a squamous epithelium due to enhanced EGF signaling (256). Overactive Notch signaling results in an abundance of secretory cells at the expense of ciliated airway cells (125, 292). Beyond the conducting zone, the epithelium of the respiratory bronchioles is composed of cuboidal cells extending to the alveolar compartment, which is lined by type I and type II pneumocytes.

Airway Innate Immune Function

The epithelial lining of the conducting airways is a key component of the airway defense against potentially damaging inhaled particles, pollutants and pathogens. The high velocity and turbulent flow of air in the proximal airways effectively filters large particles and many water soluble contaminants from the inhaled atmosphere, preventing their access to deeper regions of the lung (288). Epithelial secretions trap noxious particles and are removed and neutralized by a variety of defense mechanisms. Central to the defense function are an array of surface receptors and antimicrobial agents that constitute a major portion of the innate immune system of the airways.

The Toll-like receptors (TLRs) are critical in the recognition of microorganisms at the surface of the epithelium. TLR signaling initiates a complex signaling cascade mediated by MyD88 and results in NF-κB activation of proinflammatory genes such as IL-1β, IL-6 and CXCL8 (267). TLR signaling also regulates mucin gene expression, where direct stimulation of TLR2 and TLR3 induces mucin expression and activates MAPK and EGFR signaling (54, 417). Interestingly, mucins may have a negative feedback effect on TLR activation by negatively regulating TLR2 and TLR3 signaling (371). TLR activation also leads to Type 1 IFN signaling which results in the production of CXCR3 inflammatory chemokines that attract Th1 T cells (Type 1 T helper lymphocytes (Th1) produce IFN-γ, IL-2 and TNF-β to attract and activate cytotoxic lymphocytes (297)), linking adaptive and innate immune responses. Aside from TLRs, various other receptors, such as NOD-like receptors, TNF receptor 1, EGFR and C-type lectins, are critical components of the innate defense system which respond to pathogen-associated molecular patterns (PAMPs) and similarly converge on NF-kB activation

which induces proinflammatory genes and defense molecules such as TNF, IL-12, mucins and β-defensins.

The airway epithelium secretes a variety of antimicrobial products that are involved in the protection against inhaled and resident bacteria, viruses and fungi. These include lysozyme, lactoferrin, cationic defensins, collectins, pentraxins, CCL20, LL-37, serum amyloid A (SAA) and secretory leukocyte protease inhibitor (SLPI) (174). Many of these agents are constitutively present in the hydrating surface fluid of the airways but can also be induced in response to activation of various signaling pathways. Lysozyme, a large and abundant antimicrobial protein, targets the β1-4 glycosidic bond linking carbohydrates within peptidoglycan, a bacterial cell wall constituent and is therefore effective against gram-positive pathogens (383). Lactoferrin chelates iron and works in tandem with lysozyme to kill gram-negative pathogens by disrupting their outer membrane (89). Furthermore, both lysozyme and lactoferrin are elevated in Cystic Fibrosis and other inflammatory airway diseases, reflecting chronic inflammation (309, 364).

Airway epithelial cells are also involved with the activation and regulation of the adaptive immune response through interactions with resident dendritic cells (DCs), as well as T and B lymphocytes (174). DCs are antigen-presenting cells and are essential to the activation of T and B cells. Recruitment of additional DCs by the airway epithelium is achieved in part by the secretion of CCL20, a chemokine known to interact with CCR6 on immature DCs that also has antimicrobial properties (402). Mice lacking the CCR6 gene experience more severe lung infection following *A. fumigatus* challenge than WT, demonstrating the critical role of CCL20 secretion and interaction with DCs in the abatement of infection (273). Production of CCL20 by the airway epithelium can be induced by a number of stimuli, including TNF, IL-1, IL-4 and various PAMPs (172, 226, 274, 291).

CXCR3 ligand production and secretion by airway epithelial cells leads to the recruitment of Th1 T cells. The Th1 cytokine IFN-gamma conversely induces additional CXCR3, which recruits additional T cells and amplifies the Th1 immune response (61). The airway epithelium also produces and releases chemo-attractants for Th2 cells such as

CCL17, CCL22 and CCL1, which interact with CCR8 receptors on Th2 and Treg, or suppressor, T cells (Th2 cells evoke strong antibody responses and eosinophil accumulation; regulatory T cells (Tregs) suppress over activity of Th cells and are important in the prevention of autoimmune diseases and tolerance to allergens (67, 297)). Production of these chemokines by the epithelium can be induced by IL-4, IL-13, IFN-gamma, TGF-β as well as house dust mite allergen Der P and other PAMPs (137, 243, 360). Beyond recruitment of T cells, airway epithelial cells are able to directly interact with T cells through surface molecules such as CD40 and HLA-DR (179). Although many of these interactions lead to co-stimulation and positive feedback loops, airway epithelial cells strongly express B7-H1, a cytokine that induces Treg cells which results in a suppression of the immune response and increased antigen tolerance (72, 73, 179). While important for host defense against infection, T cell activation is heavily implicated in the pathogenesis of multiple types of inflammatory airway diseases (246, 314, 362). Hypersensitivity by the airway epithelium or chronic stimulation can lead to increased production and release of key chemokines and cytokines such as BAFF, TSLP, SLIPI and IL-33 that activate adaptive immune cells. In allergic states, epithelial innate defense mechanisms may be compromised and thus enhance the tendency of an inflammatory response and the progression of irreversible inflammatory airway disease.

Asthma

Asthma is an inflammatory disease of the airways characterized by wheezing, chest tightness and coughing due to hypersensitivity and constriction of the airways (258). Asthma affects about 25 million Americans and nearly half of them will experience a significant asthmatic episode, or attack, each year (65). Diagnosis of asthma has steadily increased since 2000, with the largest growth rate among black children (65). Prevalence and incidence of asthma among the global population is expected to rapidly expand due to increased exposure to aeroallergens and particles caused by global climate change and large-scale industrialization (74). The United States spends over $50 billion dollars annually in combined direct medical treatment and indirect costs associated with

asthma[1] (65). Asthma is, generally, an inflammatory state characterized by the infiltration of various immune cells into the airway epithelium including mast cells, eosinophils and Th2-type T cells (143). The recruitment of inflammatory immune cells is due to hyperresponsiveness by the epithelium to inhaled allergens and particles, though prevalence is not strongly correlated with general atopy (hyperallergic syndrome) (269).

Chronic secretion of inflammatory cytokines by the epithelium has been implicated in the persistence and long-term pathology of asthma and can lead to continuous cycles of epithelial damage and repair. Often, the damaged and wounded epithelium is incapable of complete restitution, leading to a chronic injury and the secretion of many growth factors (EGF, amphiregulin, TGFβ) implicated in driving structural changes and ultimately contribute to airway remodeling (70, 144, 184). Epithelial 'desquamation' is a hallmark of asthma, observed in both childhood and adult airways, suggesting aberrant repair after injury as a characteristic feature of the disease (22, 100, 199). As a result of incomplete restitution after injury, the airways of asthmatics tend to heal by 'secondary intention', forming scars and fibrotic tissue (142, 143).

The complexity and heterogeneity of the disease is often cited as a cause of incomplete and variable therapeutic responses to commonly prescribed drugs (143). Asthma, as well as COPD and other inflammatory airway diseases, are typically treated by a combination of anti-inflammatory compounds and long-acting β_2-receptoragonists (LABAs), which target airway smooth muscle to increase airway caliber by reducing intrinsic cholinergic tone (49). Likewise, long-acting muscarinic antagonists (LAMAs) are typically effective as bronchodilators in COPD patients, but less so in asthmatic patients because they are unable to block the actions of mast cell derived mediators of constriction like cys-LTs and PGD_2 (19). Common LABAs include salmeterol, formoterol and vilanterol. Bronchodilation is achieved through activation of Gs coupled adenylyl cyclases, leading to the production of cyclic-AMP (cAMP) and subsequent

[1] COPD, chronic bronchitis and emphysema accounted for more than 124,000 deaths in the United States in 2007, nearly 35 times more than asthma (2). Inflammatory airway diseases, primarily COPD caused by smoking, are the third leading cause of death in the United States, behind only cancer and heart disease (2).

activation PKA, which acts on multiple targets to decrease smooth muscle contractility. Furthermore, phospodiesterase (PDEs) inhibitors also exhibit anti-inflammatory effects, though these are mostly attributed to the attenuation of PDE activity in neutrophils, T-cells and macrophages (23, 99).

Corticosteroids have been found to be the most effective controllers of asthma and are typically administered in combination with a LABA (17). The anti-inflammatory effects attributed to inhaled corticosteroids (ICS) in asthma are multifaceted. Activation of surface glucocorticoid receptors (GR) on the airway epithelium and smooth muscle cells leads to chromatin remodeling through recruitment of histone deacetylase-2 (HDAC2) and a general inhibition of pro-inflammatory genes (161). HDAC2 activity is reduced in severe COPD and asthma, providing good evidence for this mode of action. Suppression of inflammatory transcription factors including NF-kB and AP-1 by activated GRs, as well as the upregulation of anti-inflammatory genes like IL-10, annexin-1 and the NF-κB inhibitor (IκB-α) contribute to the transcriptional effects of corticosteroids (18). Additionally, nontranscriptional effects of ICS have become increasingly recognized and it is likely corticosteroids affect protein synthesis by modulation of mRNA stability (18). The net result of these actions is reduced activation and recruitment of immune cells and thus a reduction in airway inflammation.

Beyond combined ICS/LABA therapy, a variety of additional therapeutic approaches are prescribed based upon the unique pathology of each patient's disease. In "Th2 high" airway disease, anti-IL-5 and IL-13 drugs that target these cytokines directly (blocking antibodies such as mepolizumab and reslizumab, GATA3 block) or indirectly (receptor blockers) are effective due to a primarily eosinophil associated inflammation (19). Furthermore, anti-thymic stromal lymphopoietin (TSLP) antibodies similarly reduce eosinophilia in asthmatic airways by reducing dendritic cell activation (114, 404). Additional therapeutic strategies include: CXCR2 antagonists, antibodies targeting TNF-α, IL-1 and IL-6, block of IL-17 receptor α by brodalumab, kinase inhibitors and macrolides (19). An especially difficult subtype of drug-resistant airway disease is corticosteroid resistance, characterized by enhanced p38 as well as MAPK, JNK, and ERK, kinase activity and reduced HDAC2 expression and activity. Steroid resistant

COPD is also associated with increased oxidative and nitrative stress (18). Thus, antioxidants, peroxynitrite scavengers and inhibitors of iNOS may be effective in restoring sensitivity to ICS therapy.

Mucociliary Clearance

An important component of airway host-defense involves the process of trapping and removing inspired material through mucociliary clearance (MCC). Inhaled pathogens, debris and particles that become ensnared within surface cell secretions and are removed from the airways by ciliary beating before the intrusive particles are able to interact with the epithelium. In this way, mucociliary clearance effectively suppresses chronic activation of the innate immune response. The importance of this process cannot be overstated; reduced or impaired mucociliary clearance is evident in many airway diseases, such as cystic fibrosis, COPD and primary ciliary dyskinesia, and results in chronic respiratory infections (30, 35, 39, 165, 212).

The mucociliary apparatus is composed of two distinct features: 1) a mucus trap, which rests upon 2) a thin fluid layer. Mucus is primarily composed of mucins, high molecular weight glycoproteins with complex glycosolation patterns that harbor a diverse array of carbohydrate side chains (284). Nine of the twenty known mucin (MUC) genes are expressed in human lungs (298). The dominant secreted mucins in the conducting airways are MUC5AC and MUC5B, both of which are cysteine-rich, gel-forming mucins (1). Mucins form only about 1% of healthy airway mucus, the majority (98%) of mucus mass is water. Entrapped particles are continuously removed via the mucociliary escalator to the pharynx, where they are eventually swallowed (1). In order to sustain persistent mucociliary clearance, mucins are constantly synthesized and secreted by surface secretory cells to replenish the mucus that is cleared from the airways. MUC5B is constitutively produced while MUC5AC appears to be produced in response to danger signals through an NF-κB-dependent transcription pathway (97).

The propellants of mucus on the airway surface are motile cilia, which extend from non-secretory surface cells along the lumen of the conducting airways. The cilia beat in a coordinated manner to sweep mucus along the distal to proximal axis of the

airway (1). Motile cilia are arranged in a classic '9+2' microtubule architecture, having nine outer doublets that surround a central pair (155). Dynein arms attached to peripheral microtubules generate force and motion through ATP hydrolysis (155). There are many factors that influence ciliary beat frequency including shear stress, extracellular ATP and Ca^{2+}_i mobilization, cAMP/PKA activity, as well as cGMP/PKG activity (249, 396). Recently, cilia of airway epithelia have been recognized for having a greater role in innate defense beyond driving mucociliary clearance. Cilia exclusively harbor a variety of T2R bitter taste receptors, playing a significant role in chemosensation (327). Additionally, ion channels and other receptors have been shown to localize to motile cilia, specifically K2P potassium channels in bronchial epithelium (91, 412). These findings are significant in understanding the pathology and severity of ciliopothies, in the lungs as well as in other organs.

The second feature of the mucociliary apparatus is the periciliary fluid (PCL). The thin fluid layer serves two significant roles: 1) supporting the mucin hydrogel, and 2) supplying the optimal environment for cilia beating. Mucins are hydrated immediately after exocytosis (5, 43, 378). Insufficient hydration, such as the case in cystic fibrosis (CF), results in excessively viscous mucus that is difficult to clear and results in particle stasis and potential infection. Therefore, airway water is carefully regulated by the coordinated action of anion secretion and sodium absorption, which sets the osmotic gradients for passive water flux (6, 36, 37, 113). Furthermore, the PCL contains immunoreactive proteins like Club cell secretory protein (CC-10) as well as secreted surfactant proteins A and D, indicating a role for the PCL microenvironment in defense function (66, 180). PCL height in freshly biopsied lower human airways is about 6μm in healthy subjects and about 4μm in CF patients (120). This distance (6-7μm) represents the approximate length of outstretched cilia and is the optimal depth for efficient ciliary beating (356). PCL depletion that occurs in CF is fundamental to the pathogenesis of the disease and highlights the importance of PCL height in protecting the lungs against infection.

Transcellular Sodium Absorption

Basal Na^+ reabsorption is a significant driver of PCL volume and electrolyte composition due to the concerted action of apical epithelial Na^+ channels (ENaC) and basolaterally located Na^+/K^+-ATPase (106, 145). ENaCs are, typically, heterotrimers consisting of single α, β and γ subunits (29, 166), though the more recently discovered δ subunit may substitute for α in some tissues (173). Each subunit contains two membrane-spanning domains, large extracellular domains as well as intracellular N- and C- termini (29). The three traditional ENaC subunits, as well as the δ subunit, share about 40% homology and are subject to significant posttranslational modifications such as glycosylation and proteolytic cleavage. ENaCs are ubiquitously expressed in tight (with high transepithelial resistances) epithelia throughout the body, notably distal nephron, colon and airways, and their physiologic importance is highlighted by the myriad of naturally occurring disease states caused by channel malfunction (29).

An essential posttranslational modification of ENaC is activation by proteolytic cleavage. Furin, a member of the pro-protein convertase family, increases single-channel open probability (P_o) by relieving Na^+ self-inhibition (329). Furin-mediated cleavage of α-ENaC occurs at two sites and releases an inhibitory peptide from the large extracellular loops (151, 152). Furin, as well as prostasin, also cleaves the γ subunit leading to enhanced ENaC activation (40). Addition of the cleaved inhibitory proteins to native ENaC confers similar channel inhibition, illustrating the importance of proteolytic cleavage in regulating ENaC function (45, 46). Cells endogenously expressing ENaCs contain both processed (cleaved) and nonprocessed (inactive) ENaC subunits; inactive surface channel pools may serve as a recruitable source of ENaC that can be activated by extracellular proteases (92, 185). Protease inhibitors, such as protease nexin 1 (PN-1) (384), represent another level of channel regulation. PN-1 expression is regulated by aldosterone and is an endogenous inhibitor of prostasin that leads to enhanced natriuresis by limiting ENaC activity (384). This suggests a complex and coordinated cellular balance between channel activating and inactivating proteins. Interestingly, improper protease balance may contribute to CF pathogenesis (29).

Another fundamental component of ENaC regulation is channel ubiquination by Nedd4-2 protein ligase (342). Surface ENaCs are subject to persistent endocytosis events that limit the amount of functional channels within the apical membrane. ENaC ubiquination is necessary but not sufficient for channel internalization (169, 413) and serves to mark the channel for degradation. Disruption of Nedd4-2 ubiquination, such as gain-of-function mutations in the C-terminal of β or γ subunits (Liddle's syndrome) (331), removes a potent inhibitory mechanism of sodium transport and results in treatment resistant hypertension (167). Regulation of Nedd4-2 ubiquitin ligase activity occurs through a variety of mechansims including phosphorylation, changes in intracellular Na^+ concentration and hormonal control via aldosterone (29). Nedd4-2 phosphorylation by serum and glucocorticoid kinase 1 (SGK1) is a well-studied regulatory process that limits Nedd4-2 interaction with ENaC (80). Furthermore, SGK1, as well as other protein kinases (IkB, PKA, PKD), can directly phosphorylate ENaC leading to enhanced channel activity (82, 206, 235, 338). On the other hand, PKC decreases ENaC surface expression and P_o while ERK2 phosphorylation of the β and γ subunits promotes Nedd4-2 mediated internalization (330). The complex ENaC regulatory web, with layers of redundant pathways, demonstrates the importance of strict sodium handling and highlights the physiologic significance of ENaCs.

ENaC is the principal target of the potassium-sparing diuretic amiloride (173). Amiloride inhibition occurs primarily by ionic block of the channel, though allosteric effects have been suggested (25, 131). The second generation amiloride analog benzamil has a substituted benzyl group on the terminal nitrogen atom of the guanidine moiety of amiloride (141), resulting in 9-fold greater potency. Amiloride-sensitive basal sodium absorption has been observed in human airway epithelium as well as many other mammalian species (14, 217), and plays a significant role in regulating PCL height. The driving force for basal Na^+ reabsorption through ENaC is maintained by the constitutive efflux of Na^+ across the basolateral membrane by the Na^+/K^+-ATPase (71, 85). The Na^+/K^+-ATPase consists of a catalytic α subunits and regulatory β and γ subunits (373). The dominant α isoform in the pulmonary epithelium is α_1 (96). The Na^+/K^+-ATPase is regulated by a variety of mechanisms including PKA phosphorylation (281), hormonal

steroid control (311), and increased intracellular Na^+ concentration (326). Na^+/K^+-ATPase is inhibited by ouabain and is the target of cardiac glycosides.

Transcellular Chloride Secretion

Although all airway epithelia (nasal, trachea, bronchial) are classically described as absorptive (145), anion secretion, primarily Cl^- and HCO_3^- ions, is essential for the regulation of PCL viscosity and depth. A large component of anion secretion in the conducting airways occurs through apically located cystic fibrosis transmembrane conductance regulator (CFTR) channels (324). CFTR belongs to the ATP-binding cassette (ABC) transporter family of proteins, although it functions as an ion channel not a transporter (140, 377). CFTR consists of two transmembrane domains, each spanning the plasma membrane six times, two nucleotide binding domains (NBDs) and an extended hydrophilic domain immediately following NBD1, known as the R-domain (377). Phosphorylation of the R-domain at several distinct but redundant PKA sites is necessary for NBD interaction with ATP (263). Thus, activation of adenylyl cyclases through signaling cascades or by inhibition of phosphodiesterases, leading to an elevation of intracellular cyclic-AMP (cAMP), results in CFTR activation *in vivo* and *in vitro*. However, CFTR activation occurs only after phosphorylation *and* ATP binding. ATP hydrolysis at NBD2 occurs much more rapidly than NBD1 (3) and appears to be the more significant NBD with regards to channel gating; K1250A mutations prevent binding and hydrolysis of ATP at NBD2 and correlates with greatly reduced channel- deactivation kinetics, while K464A mutations to NBD1 have a very limited effect on channel opening (3).

CFTR is highly selective for monovalent anions (21) with the following observed permeability sequence: $NO_3^- > Cl^- > HCO_3^-$ > formate $^-$ > acetate$^-$ (210, 211). Iodide (I^-), a lyotropic anion, binds the channel tightly and inhibits transport of large, weakly hydrated anions like Cl^-, though P_I/P_{Cl} ratios tend to be inconsistent and may depend on species specific sequences of transmembrane domains 1-6 (283, 350). The single channel CFTR conductance for Cl^- is 7 to 10pS but can be reduced to 2pS with elevated SCN^- mole fraction (351). CFTR exhibits anomalous mole fraction behavior and the single

channel conductance recovers when SCN^- represents 97% of soluble anion (Cl^- /SCN^- mixtures). Recent high-throughput screening of small-molecules has resulted in the discovery of several classes of CFTR inhibitors, including the thiazolidinone ($CFTR_{inh}$-172), glycine hydrazide (GlyH-101) and quinoxalinedione (PPQ-102, BPO-27) chemical classes (382). $CFTR_{inh}$-172 inhibits CFTR Cl^- conductance with an IC_{50} of 0.5-4μM based on short-circuit current measurements (215), and functions as a closed-channel stabilizer with no effect on the CFTR current-voltage relationship (352).

The CFTR channel is the product of the gene that is mutated in the autosomal recessive disease cystic fibrosis (CF) (57). CF patients suffer from persistent lung infections and dramatically reduced life expectancy. More than 30,000 people are living with CF in the United States, with approximately 1,000 new cases a year (104). Although there are more than 1,800 known genetic mutations that cause the disease, the most common is the ΔF508 mutation, the deletion of a single phenylalanine residue that causes the full-length glycosylated molecule to remain in the ER due to defective folding of the polypeptide (232, 286). The result is very little mature CFTR delivered to the cell surface in all tissues. Interestingly, CF symptoms in the lung may not simply be due to abnormally low Cl^- secretion but rather the loss of CFTR interactions with and on other proteins, such as ENaC, as evident by Na^+ hyperabsorption in CF patients (232). Treatment strategies for CF patients include hypertonic saline, mucolytic agents, antibiotics, combined inhalers and other quality of life maintenance strategies. More recently, molecules that promote maturation of ΔF508 CFTR have been introduced to the market (Lumacaftor (VX-809)). Two classes of drugs have been developed: 1) potentiators such as PG-01 and VX-770, and 2) correctors like Corr-4a and VX-809 (303). Several clinical trials investigating the efficacy of both drug classes have revealed nominal improvement in CF patients, with the most promising drug being the CFTR potentiator ivafaftor (303). A limitation in clinical drug efficacy is understandable since drug discovery focuses simply on restoring channel function, and not on the role CFTR plays within the entire cellular environment, due to the limitations of preclinical screening assays (303).

Besides CFTR, there are numerous other anion channels expressed by airway epithelial cells. There are several calcium-activated chloride channels. The best studied among them is TMEM16a (ANO1), although this channel accounts for only a small portion of total calcium-activated anion currents (252, 294). TMEM16a has garnered significant attention as a surrogate chloride channel in CF patients, where channel activation could potentially abrogate the loss of PCL volume seen in CF (339). However, TMEM16a has been shown to play a significant role in asthmatic mucous cell hyperplasia and airway smooth muscle (ASM) responsiveness as demonstrated by reduced mucin secretion and ASM contraction in cells and tissues exposed to channel inhibitors (150).

Cellular loading of Cl^- across the basolateral membrane is achieved in an electroneutral manner by $Na^+/K^+/2Cl^-$ cotransporters (NKCC) (361). NKCCs are inhibited by loop diuretics, such as bumetanide and furosemide (162), and deliver a continuous supply of Cl^- due to the inward electrochemical gradient for Na^+ (105). Reduced cellular Cl^- concentration results in WNK (With No Lysine Kinase) activation and eventual phosphorylation of the cotransporter, inducing greater ion turnover (146). Thus, the overall rate of Cl^- secretion is determined by the rate of entry provided by NKCC across the basolateral membrane (105). NKCCs play significant roles in regulating cellular volume and this process has been shown to be regulated N-terminal phosphorylation by SPAK (surface presentation of antigens protein) and OSR1 (oxidative stress responsive 1) (237).

Potassium Channels involved in MCC

NKCC K^+ uptake is partially recycled across the basolateral membrane, which maintains the electrochemical gradient for apical anion secretion (145). Basolateral K^+ recycling from the cell to the unstirred fluid layer adjacent to the basolateral membrane is essential in sustaining Na^+-K^+ ATPase. Furthermore, K^+ secretion across the basolateral membrane offsets the membrane depolarizing effects of Na^+ absorption and anion secretion and is therefore an essential component of mucociliary clearance. Channels involved in this process include $K_v7.1$ and $K_{Ca}3.1$ potassium channels (28, 219). Airway

epithelial cells express more than 30 different K^+ channels belonging to three main classes: six-transmembrane domain channels, both voltage and calcium-activated, two pore channels, and inward rectifiers (15). Significant channels associated with control of Cl^- secretion are $K_v7.1$ voltage-gated K^+ channels and $K_{Ca}3.1$, intermediate conductance, potassium channels. Inhibition of both of these channels significantly reduced Cl^- secretion in tracheal and bronchial epithelial cells (28, 68, 81, 218, 219). Conversely, $K_{Ca}3.1$ activation stimulates Cl^- secretion through CFTR and CaCCs in healthy airway cells (218, 334, 335, 349).

Recently, apical $K_{Ca}1.1$ (BK_{Ca}), or large conductance, K^+ channels have been identified as being a critical player in the maintenance of PCL volume (223). BK channels have a large single channel conductance (100-300pS) and are activated in response to membrane depolarization and elevated intracellular Ca^{2+} (207). They are expressed in multiple tissues throughout the body, including neurons and smooth muscle, and achieve functional diversity through alternative gene splicing and association with modulatory auxiliary subunits (207). There are four known β subunits as well four recently discovered γ subunits, belonging to the leucine-rich repeat-containing (LRRC) protein family (183). The β subunits are implicated in multiple control mechanisms, especially those that alter BK sensitivity for calcium (195). LRRC26, a γ subunit expressed in airway epithelium, shifts the voltage dependence of BK activation towards less depolarized potentials, allowing channel activity in nonexcitatory cells (410). Mutations in the BK genes are associated with epilepsy (86, 213) and channels are the target of many dangerous natural toxins, such as iberiotoxin and paxilline, which inhibit after-hyperpolarization currents, disrupting neuronal signaling (347). In airways, paxilline-sensitive currents facilitate threefold greater Cl^- efflux (223) and long-term inhibition of BK potassium flux leads to airway surface dehydration and reduced ciliary beat frequency (223).

Important Signaling Pathways in MCC

Cellular modulation of transepithelial ion transport pathways occurs in many ways (post-translational modification, phosphorylation, slice-variation, associated

channel subunits, etc.). External autocrine and paracrine signals, as well as environmental agonists, influence net ionic movement through activation of specific surface-receptor mediated pathways. β_2-adrenergic receptors (β_2-ARs) for example, are G_s coupled GPCRs that are endogenously activated by epinephrine. Stimulation of these receptors results in PKA activation and modulation of various physiologic pathways including smooth-muscle relaxation and increased cardiac output. Previous studies have demonstrated that β_2-ARs form a signaling complex with CFTR in the apical membrane of epithelial cells (253). Stimulation of airway epithelial cells with β_2 agonists leads to elevated cellular cAMP and CFTR activation (271). Calu-3 cells, a human adenocarcinoma cell line derived from airway submucosal gland cells, stimulated with epinephrine or salbutamol exhibit significantly delayed cell migration, suggesting a prominent role of CFTR in airway restitution following injury (270). Another significant autocrine signaling pathway involves P2Y purinergic receptors and P1 adenosine receptors (42). The effect of purinergic agonists, acting specifically at $P2Y_2$ receptors, on transepithelial ion transport in the airways has been well documented (42, 94, 145, 192). P2Y receptors are G-protein coupled receptors and mobilize $Ca^{2+}{}_i$ through PLC mediated IP_3 production. ATP and UTP acting at $P2Y_2$ receptors in bronchial epithelial cells have been shown to increase Cl^- efflux through calcium activated channels as well as increase K^+ efflux through calcium sensitive K^+ channels (218, 306, 319). $P2Y_2$ receptor mediated $Ca^{2+}{}_i$ mobilization can also activate calcium sensitive adenylyl cylase leading to PKA mediated phosphorylation of CFTR and additional Cl- efflux. Furthermore, P2Y-R agonists inhibit ENaC dependent Na^+ absorption and thus have been developed as a therapy for cystic fibrosis since excessive Na^+ uptake leads to severely impaired mucociliary clearance (192, 193). P2Y-R agonists also increase ciliary beat frequency and mucous secretion (55, 135, 394).

Adenosine effects on airway epithelia have been equally well characterized (64, 186, 289, 388). Extracellular ATP is broken down by multiple enzymes including ecto-ATPases (NTPDase3 and ecto-5'NT), which ultimately generate adenosine within the PCL that in turn binds to A_{2B} receptors at the membrane surface. These receptors increase cAMP through activation of adenylyl cyclase. cAMP then activates protein kinase A,

which phosphorylates CFTR and increases anion secretion (153). In this manner, adenosine can act to increase PCL height without mobilizing Ca^{2+} and without disruption of ENaC dependent sodium absorption.

Transcellular Water Transport

Ion transport by the pulmonary epithelium produces an osmotic gradient that drives fluid secretion and absorption that ultimately determines the volume of the PCL. Biophysical studies of whole-lung preparations have revealed relatively high water permeability, consistent with transcellular water transport through aquaporin water channels (AQPs) (188). AQPs are proteins that facilitate bidirectional, single-file transport of water (379). Because of their limited single-channel capacity, AQPs are present in membranes at very high density (1000s/μm^2 vs. <10/ μm^2 for ion channels) (401), which allows up to 50-fold higher osmotic water permeability than membranes without AQPs (380). The airway epithelium expresses AQP3, 4 and 5, with differential localization depending on position along the pulmonary tree (188). AQP3 is also capable of transporting glycerol and studies have demonstrated CFTR-dependent regulation in airway epithelial cells (320). AQP3-dependent glycerol transport promotes cell proliferation in other tissues but this potential role has not yet been investigated in airway epithelium. Nonetheless, loss of CFTR-mediated AQP3 function may impair cellular restitution in CF, as well as impeding water flux, and may play a significant role in CF pathology. Besides transcellular water transport, passive flux of water, ions and macromolecules occurs through a regulated paracellular pathway.

Paracellular Pathway

Intercellular, epithelial-epithelial junctions form important connections between adjacent cells and maintain a physical barrier that separates the external from the internal environment (39). The paracellular space is characterized by three predominant structures: tight junctions, adherens junctions and desmosomes (279). The desmosomes are the most distal adhesive structure from the luminal surface where they directly connect to cellular intermediate filaments and resist damaging forces produced by

mechanical stress that could cause separation of cells within the epithelium (39). Adherens junctions are composed of E-cadherin and a multitude of catenin family proteins, including α-catenin, β-catenin and p120. These structures anchor to the actin cytoskeleton and are mediators of cell-cell adhesion (98, 133, 164). Cis E-cadherin interactions require Ca^{2+}, which stiffens the extracellular domain of the integral-membrane protein (20). Adherens junctions also play an important role in facilitating cell signaling due to their capacity to sequester catenin proteins. β-catenin is a key contributor to canonical Wnt signaling pathway (367), where cytoplasmic β-catenin translocates to the nucleus and functions as an activator of genes involved in adhesion, morphogenesis, epithelial-to-mesenchymal transition, de-epithelization and tumor development (60, 121). Sequestration of β-catenin at the intracellular domains of adherens junctions effectively reduces available β-catenin to participate in Wnt signaling (367). Therefore, adherens junctions limit Wnt-mediated signaling and promote epithelial stability (20).

Epithelial tight junctions are the most apically located of the three adhesion structures. They are multiprotein complexes composed of transmembrane proteins including occludin, junctional adhesion molecules (JAMs), and claudins, which are anchored internally to actin through scaffolding proteins like ZO-1 (39). Tight junctions (TJs) form a continuous ring about the circumference of the epithelial cell and are the critical structure in regulating paracellular ion, water and molecular transport (133, 318). The paracellular pathway is composed of aqueous pores ranging in size from 7 to 15Å in radius (374). These pores show relatively low discrimination for similarly charged but differently sized cations, as opposed to membrane bound channels, which can show thousand-fold preference (282). TJs are not gated, highly dynamic and are involved in the control of two distinct modes of transepithelial transport. The first is the facilitation of paracellular transport of small electrolytes that is dependent on properties of the paracellular pathway that contribute to its transepithelial electrical resistance (TER) (374). The second is the transport of larger, non-charged molecules, like mannitol and glucose, which is measured over minutes and hours using flux tracers. An example of the importance of the paracellular pathway in the transport of small solutes is intestinal

glucose absoption. Transcellular glucose transport greatly exceeds the capacity of intestinal SGLT1, Na^+-glucose cotransporters, and cells respond to elevated luminal glucose by contracting the perijunctional cytoskeleton to allow for greater paracellular permeability (216, 370). Paradoxically, many studies have described a decrease in electrical conductance while simultaneously increasing flux of large, uncharged solutes (234). A likely explanation of this phenomenon is frequent breaking then re-sealing of intercellular TJ strands, which allows for slow transit of large molecules while maintaining TER.

Claudin proteins have emerged as the key determinant of TJ charge and size selectivity (265). First discovered as a strand-forming component of TJs in 1998 (108), there are currently 27 members of the claudin gene family that encode 22-27 kDa proteins (123). Claudins contain four transmembrane domains and two extracellular loops that interact with adjacent claudins in both homotypic and heterotypic binding (187). Epithelial, as well as endotheial, cells express claudins in a tissue-specific manner, allowing for remarkable variability in TJ composition (265). Claudin expression is a key determinant of overall tissue permeability; such is the case with “leaky” renal epithelia such as the proximal tubule, or the ”tight”, relatively impermeable blood-brain barrier (BBB) microvasculature (124, 372). The pulmonary epithelium expresses at least 14 claudin mRNAs (187), of which the best studied are claudin-3 and claudin-4 due to their abundant expression in the alveoli (187). Increased expression of claudin-3 in type II pneumocytes is associated with an increase in alveolar permeability (241). This highlights the bimodal effect that any single claudin can have on the TJ. Categorically, claudins are characterized as either pore-forming (increase permeability) or sealing (reduce permeability). Pore-forming claudins, such as claudin-2, claudin-7 and claudin-10, are typically expressed by “leaky” epithelial tissues. Claudin-2 has been shown to form pores specific for monovalent cations and water in MDCK cells (4, 299). Barrier forming claudins, such as claudin-1, claudin-5 and claudin-11, are generally considered sealing though this function is not universal (265). For example, claudin-5 is a necessary component of BBB TJs but, when overexpressed in airway and alveolar epithelial cells, leads to a decrease in TER (69, 76). Thus, it is likely more important to consider the

entire ensemble of claudins expressed by a tissue when determining function and it is difficult to describe them in binary terms since individual claudin contributions to permeability depend heavily on interactions with other components of the TJ microenvironment.

Claudins are also implicated in the pathology of several human diseases. Mutations to claudin-14 results in nonsyndromic deafness due to cation leak between the perilymph and endolymph in the cochlea of the ear (24). Familial hypomagneaemia with hypercalciuria and nephrocalcinosis (FHHNC), an autosomal recessive disorder that results in divalent cation wasting and chronic renal failure, has been linked to mutations in the claudin-16 gene (132). Paracellular absorption of magnesium and calcium in the thick ascending loop of Henle is achieved through the maintenance of a positive luminal potential relative to the serosal membrane (32). Claudin-16 interaction with claudin-19 create TJ pores with increased selectivity to sodium which permits development of an electrochemical gradient favorable to Mg^{2+} and Ca^{2+} reabsorption (148). Reduced cation selectivity in the TJs caused by a claudin-16 mutation abolishes the driving force for divalent uptake from the filtrate and ultimately leads to their loss in the urine.

Aside from acting as a selective barrier to solute movement (barrier function), TJs serve as a fence to mechanically restrict the free diffusion of membrane proteins and lipids within the plasma membrane (83). This role of TJs ensures the partition between apical and basolateral membranes, an essential distinction required for transepithelial transport (123). Depolymerization of the circumferential actin network leads to disruption of barrier but not fence function of TJs, suggesting independent functional mechanisms (221, 354). Moreover, recent experiments suggest claudins are not required for TJ fence function and thus are not essential for epithelial polarization (10, 156, 242).

Electrophysiologic Measurements

Functional characterization of epithelial tissues using electrophysiologic techniques is well established. Transepithelial resistance (TER), an electrical property indicative of TJ permeability and overall transepithelial conductance, can be measured with chopstick electrodes and a voltohm meter. An STX2 electrode (World Precision

Instruments, Sarasota, FL) consists of four components, two silver (Ag) pads that pass current and two Ag/AgCl pads that sense voltage. An AC current of known magnitude is passed between the two ends of the chopstick electrode with the epithelial tissue between the electrodes. Then, using Ohm's Law and the potential between the two voltage sensing pads, the resistance can be calculated. Another important technique used to characterize paracellular permeability include bi-ionic and dilution potential experiments. In a bi-ionic experiment, ions of similar charge but different size are introduced to a sample in an asymmetric manner. This sets up a chemical diffusive gradient across the epithelial tissue. A bi-ionic experiment allows for analysis of size selectivity through the paracellular pathway as well as informing a rank order of permeability for ionic species. Dilution potential measurements involve the spontaneous development of a transepithelial potential difference following an abrupt change in ion concentrations across the epithelium.. For example, epithelia bathed in asymmetric NaCl solutions will instantaneously generate a dilution potential that is indicative of paracellular permeability and charge selectivity. Since dilution potentials are rapid phenomena, appropriate sampling must be considered. One method that allows for continuous transepithelial potential measurement is voltage-clamping an epithelial tissue in an Ussing chamber.

The Ussing chamber is a device developed by the Danish zoologist Hans Ussing in 1946 to measure transport of charged solutes across an epithelium (201). A polarized epithelial tissue is used to divide a single chamber into two compartments. When the tissue is bathed in a symmetrical saline solution, a spontaneous potential difference arises across the epithelium due to transepithelial movement of ions. Short circuit current (I_{SC}), or 'shunt' current, is defined as the charge flow per time when the tissue is short-circuited, and is equivalent to the net flux of all electrogenically transported solutes across the epithelium (208). Dissection of I_{SC} into individual ionic currents can be achieved pharmacologically by adding transport inhibitors or by replacement of ions with impermeable solutes. Similarly, increased channel activity is also reflected in I_{SC} measurements following addition of receptor agonists or cell permeable modulators to the bath solutions. Ussing chambers are very powerful tools for probing ion transport

processes in epithelial tissues but require significant understanding of underlying physiological processes for proper interpretation of measurements (208).

In Vitro Differentiation of Normal Human Bronchial Epithelial cells

Airway epithelial cells can be grown in standard, submersed tissue culture. Primary cultures isolated from the conducting airways are composed of almost entirely basal epithelial cells rather than surface epithelial cells, due to their adhesive properties and capacity to replicate (385). Basal cells cultured under submersed, 2-dimensional conditions assume a squamous phenotype and do not recapitulate *in vivo* airway biology, specifically pulmonary epithelium transport properties. However, when grown using appropriate culture conditions, airway basal cells are capable of forming a ciliated pseudostratified epithelium that can be used to investigate native epithelial function (119, 168, 392). Airway basal epithelial cells (NHBE) grown with an air-liquid interface (ALI) on porous transwell filters become polarized and undergo mucociliary differentiation. During the differentiation process these monolayers develop transepithelial resistances (TER) consistent with the formation of tight junctions (TJs) that regulate the permeability of the paracellular pathway. These monolayers contain basal cell-derived ciliated and mucus-producing surface cells as well as basal epithelial cells. Furthermore, NHBE cells differentiated in this manner demonstrate vectoral mucus transport, resistance to gene therapy viruses, and cell-type specific viral infection (232, 233, 392, 411).

Culture media is equally important for NHBE growth and differentiation. NHBE cells are maintained in low Ca^{2+} containing media during expansion. Both TJs and adherens junctions are Ca^{2+} dependent. Maintaining the NHBE in low or Ca^{2+}-free media prevents junctional formation and promotes replication driven in part by Wnt/BETA-catenin signaling (181). TER dependence on Ca^{2+} has been well documented and has been utilized in Ca^{2+}-switch experiments to characterize the nature of the paracellular barrier (12, 50, 117, 228). Bronchial epithelial growth medium (BEGM) can be purchased commercially and typically contains the following additives: bovine pituitary extract, insulin, transferrin, triiodothyronine, epinephrine, epidermal growth factor,

hydrocortisone, retinoic acid, antibiotics and antifungal agents. NHBE differentiation media is nearly identical to BEGM. One difference is that differentiation media possesses a physiological [Ca^{2+}], containing at least 1mM $CaCl_2$ (119, 307). Differentiation media also contains an elevated concentration of retinoic acid (RA), a vitamin A metabolite, which is essential to suppress squamous metaplasia in culture (405). NHBE differentiation in the absence of RA results in a stratified squamous epithelium with reduced mucin secretion (168, 307, 392). RA acts to prevent basal cell hyperplasia by suppressing the expression of cytokeratins and is used as a topical treatment in certain corneal and epidermal diseases (59, 139, 200) . NHBE cells grown in differentiation media but not with an ALI still undergo differentiation but do not produce ciliated cells, since submersion blocks ciliogenesis *in vitro* (264).

NHBE differentiation following the protocols used in this thesis decidedly recapitulates *in vivo* behavior of the airway epithelium. Differentiated monolayers display innate immune function, transepithelial transport, intact signaling pathways and polarized distribution of membrane lipids and proteins. This gives investigators a unique opportunity to probe native airway epithelial physiology in a controlled environment without confounding contributions from the mesenchyme, nervous system or adaptive immune system. However, this is also a limitation of the approach since it is only the NHBE cells that produce the differentiated, pseudostratified tissue. Nonetheless, this mucociliary differentiation procedure has been utilized to study the transcriptional events and cues that occur during basal cell differentiation (300), the effects of inflammatory cytokines on structure and function of the pulmonary epithelium (224, 225), and the differences between normal and diseased airway epithelia such as asthma and cystic fibrosis (363, 408).

The present research utilized *in vitro* NHBE differentiation techniques to study the effects of hydrocortisone (HC) on the development and maintenance of airway epithelium transport processes during mucociliary differentiation. HC is a corticosteroid that is classified as a glucocorticoid, which is distinct from a second class of corticosteroids known as mineralocorticoids. Corticosteroids are synthesized from cholesterol in the adrenal cortex (268). The primary mineralocorticoid is aldosterone,

which acts to regulate sodium balance through regulation of transport mechanisms in the nephron. Aldosterone release into the blood stream is stimulated by the renin-angiotensin system in response to low plasma sodium. Aldosterone strongly regulates the expression and activity of ENaC expressed in the aldosterone sensitive distal nephron (ASDN) through transcriptional control after binding and internalization of surface mineralocorticoid receptors (MRs) (302). Aldosterone activated MRs enter the nucleus where they act as transcription factors at hormone response elements (HREs) in the genome (302). Aldosterone directly modulates the expression of ENaCs as well as channel regulators such as SGK1, GILZ1 (a scaffolding protein involved in Nedd4-2 ubiquitination), and CNK3, which combine to form a protein complex that increases cell surface expression and activity of ENaC (340). Aldosterone has a similar effect on the Na^+/K^+-ATPase, where it increases both the surface density and activity of the pump (311).

Glucocorticoids also modulate a diverse assortment of physiologic functions. Their name derives from the role they play in glucose metabolism, specifically in maintaining normal glucose concentrations in blood by stimulating gluconeogenesis during periods of fasting (194). Activated glucocorticoid receptors (GRs), in a similar manner as MRs, enhance the expression of enzymes required for gluconeogenesis (194). Antinetal administration of glucocorticoids is the standard of care to reduce neonatal morbidity and mortality from preterm birth. Glucocorticoids rapidly mature the pulmonary epithelium, converting the lungs from a secretory to absorptive tissue able to exchange gases ex utero. Antenatal steroids enhance development of type I and II pneumocytes leading to increased surfactant production, antioxidant enzyme production and increased lung volume and compliance (13, 337, 386). Furthermore, glucocorticoid regulation of ENaC enhances reabsorption of pulmonary fluid shortly before birth in response to circulating catecholamines (229, 276). Antenatal administration of glucocorticoids greatly reduces the risk of neonatal respiratory distress syndrome (260).

Corticosteroids also play an important role in suppressing inflammation in airway diseases like asthma, COPD and CF. Inhaled corticosteroids (ICS) activate surface GRs on airway epithelial cells, leading to translocation to the nucleus where they suppress pro-

inflammatory transcription factors like NF-κB and AP-1 (343). Similarly, GRs can indirectly enhance expression of important host defense proteins such as TLR-2. GRs induce the expression of MAPK phosphatase 1 (MKP-1) which inhibits p38 MAPK negative regulation of TLR-2 transcription (157, 333). MKP-1 is a primary effector of glucocorticoid posttranscriptional regulation (202). Examples include repression of pro-inflammatory cytokines and chemokines (IL-1β, TNF-ALPHA, CXCL10, TGF-β, β-defensins), as well as regulating mRNA transport, stability and translation (343, 344). mRNA regulation by glucocorticoids also occurs through transcription of tristetraprolin, an early inflammatory response gene that binds mRNA and promotes decay of inflammatory transcripts like TNF-α, COX-2, and iNOS (47, 48).

Glucocorticoid suppression of inflammatory cytokines is important in the regulation of MCC and the maintenance of the PCL depth. IFN-γ is an inflammatory cytokine secreted by T-cells that activates macrophages (321). Studies of airway epithelial cells exposed to IFN-γ revealed reduced benzamil-sensitive Na^+ absorption, enhanced Ca^{2+}-activated Cl^- secretion, suppression of BK K^+ secretion and a reduced ciliary beat frequency (110, 225). IL-4, an inflammatory cytokine produced by airway epithelial cells, similarly reduced Na^+ absorption and increased anion secretion, though by a cAMP stimulated current (75, 111). Airway epithelial cells exposed to TGF-β had a reduced BK K^+ secretion, though its effect on ENaC Na^+ absorption is varied (109, 224). These results demonstrate the importance of glucocorticoid suppression of inflammatory cytokines and cheomkines with regards to ion transport and MCC.

The transcriptional effects of glucocorticoids on airway epithelial cells have been well studied and mirror many of the observed effects of aldosterone on renal epithelium. Pioneering work on the effects of corticosteroids on pulmonary epithelium demonstrated that dexamethasone, a synthetic glucocorticoid, as well as aldosterone increased the amiloride-sensitive current in rat airway cells after just 10 hours of exposure (52). Dexamethasone has also been shown to enhance transcription of α, β, and γ-ENaC in H441 and A549 airway epithelial cell lines (160, 205, 313). Dexamethasone enhancement of ENaC Na^+ absorption is linked to activation of SGK1, though only transiently (390). Glucocorticoids also allowed for greater cAMP-dependent control of

PKA over Nedd4-2, resulting in greater ENaC surface density and enhanced Na^+ absorption (159). Although these studies are important in linking corticosteroids to transcriptional control in airway epithelial cells, these studies were all conducted on cancer and immortalized cell lines that do not differentiate into a pseudostratified, mucociliary epithelium. Furthermore, these experiments exposed airway epithelial cells to corticosteroids in an acute manner and do not necessarily reflect the long-term effects of corticosteroid on transepithelial transport. Therefore, there exists a gap in knowledge regarding the effects of corticosteroids on the mucociliary differentiation of airway basal cells and the development of the ion transport phenotype of bronchial epithelial cells.

Objective and Specific Aims

The main goal of this thesis project was to characterize the effects of corticosteroids on the mucociliary differentiation of airway basal cells and the development of the ion transport phenotype of bronchial epithelial cells. The majority of what is known about the effects of corticosteroids, specifically glucocorticoids, on airway epithelial cells has been studied on cancer or immortalized cell lines that do no recapitulate the pseudostratified and mucociliated epithelium characteristic of the conducting airways. The studies presented in this thesis focus on the effects of glucocorticoids during a multi-week mucuciliary differentiation of normal human bronchial epithelial cells that possess characteristics of airway basal cells. Three specific aims were developed to address the main goal of this thesis:

Aim 1) Determine the role of hydrocortisone during mucociliary differentiation of airway basal cells.

Aim 2) Investigate the effects of hydrocortisone on basal transepithelial ion transport processes required for mucociliary clearance and determine the role of hydrocortisone on specific signaling pathways critical to the regulation of electrolyte absorption and secretion.

Aim 3) Characterize the paracellular transport pathway of the differentiated airway epithelium and determine the effects of hydrocortisone on the paracellular ion permeability.

In Chapter 2, the role of HC on the differentiation and basal sodium and anion transport in well differentiated NHBE tissues is presented. The effects of HC on the coupling of β_2-AR with CFTR was also determined and compared to results obtained with differentiated cystic fibrosis bronchial epithelial cells possessing the ΔF508 mutation. In Chapter 3, the effects of HC on UTP-stimulated anion and cation fluxes were measured, with particular focus on large-conductance calcium-activated potassium

channels. Finally, in Chapter 4, a characterization of the paracellular pathway in differentiated NHBE cells, highlighting the claudin expression profile during mucociliary differentiation and how differentiation affects the ion permeability properties of the tight junction, which is necessary in supporting transepithelial ion transport. A common theme throughout the thesis concerns the importance of motile cilia as a unique membrane domain that harbors specific ion channels and receptors. The significance of ciliary localization of these transport/signaling complexes in the context of mucociliary clearance is discussed in the concluding discussion section.

Chapter 2

Differentiation of human bronchial epithelial cells: Role of hydrocortisone in development of ion transport pathways involved in mucociliary clearance

Nathan A. Zaidman[†], Angela Panoskaltsis-Mortari[†*] and Scott M. O'Grady[†§]

Departments of Integrative Biology and Physiology[†], Pediatrics[*], and Animal Science[§], University of Minnesota, 6-125 Jackson Hall, Minneapolis MN.

American Journal of Physiology - Cell Physiology 311.2: C225-C236

Synopsis

Glucocorticoids strongly influence the mucosal-defense functions performed by the bronchial epithelium and inhaled corticosteroids (ICS) are critical in the treatment of patients with inflammatory airway diseases such as asthma, COPD and cystic fibrosis. A common pathology associated with these diseases is reduced mucociliary clearance, a defense mechanism involving the coordinated transport of salt, water and mucus by the bronchial epithelium, ultimately leading to the retention of pathogens and particles in the airways and to further disease progression. In the present study, we investigated the role of hydrocortisone (HC) in differentiation and development of the ion transport phenotype of normal human bronchial epithelial (NHBE) cells under air-liquid interface (ALI) conditions. NHBE cells differentiated in the absence of HC (HC0) showed significantly less benzamil-sensitive short-circuit current compared to controls as well as a reduced response after stimulation with the selective β_2-adrenergic receptor (AR) agonist salbutamol. Apical membrane localization of ENaCα subunits were similarly reduced in HC0 cells compared to controls, supporting a role of HC in the trafficking and density of sodium channels in the plasma membrane. Additionally, glucocorticoid exposure during differentiation regulated the transcription of CFTR and β_2-AR mRNAs and appeared to be necessary for the expression of CFTR-dependent anion secretion in response to □- agonists. HC had no significant effect on surface cell differentiation but did modulate the expression of mucin mRNAs. These findings indicated that glucocorticoids support mucosal defense by regulating critical transport pathways essential for effective mucociliary clearance.

Introduction

The airway epithelium establishes a boundary between the internal and the external environment that protects against potential injury and infection caused by inhaled particles, debris and microbial pathogens (77, 267, 300). In order to maintain the health of the lungs and surrounding tissues, the epithelium utilizes numerous defense mechanisms that complement the barrier function of the epithelium. These include the expression of a broad array of innate immune receptors including Toll-like, NOD-like and RIG-I receptors which facilitate the expression and secretion of multiple defense molecules, cytokines and chemokines essential for recruitment and activation of immune cells (174, 267). Furthermore, the airway epithelium produces antimicrobial agents such as reactive oxygen species, defensins, iron chelating proteins, and interferons that effectively prevent infection by inhaled pathogens without activation of an adaptive immune response (174, 267). Chronic inflammation of the airways due to prolonged or repeated exposure to noxious agents can result in epithelial remodeling and fibrosis that leaves the airways more susceptible to infection due to disruption of innate immune processes (280, 322, 353). Loss of epithelial integrity compromises normal mucosal defense, underscoring the important relationship between structure and function of the airway epithelium (130, 285).

The pseudostratified airway epithelium is composed of multiple cell types (288). Multipotent, progenitor basal cells are transit-amplifying cells that reside along the basal lamina of the airway which differentiate into surface cells (128). Basal cells are instrumental in the maintenance of normal epithelial structure and function as well as driving orderly regeneration after injury (293, 296). Differentiated surface cells can be divided into two distinct lineages: secretory and ciliated. Secretory cells synthesize and secrete gel-forming polymeric mucins that absorb water to form mucus (1, 189). Ciliated cells propel the mucus gel towards the pharynx and out of the lungs by the directional, synchronized beating of cilia on the cell surface, effectively removing entrapped particles and pathogens from the lungs by a process known as mucociliary clearance (220, 325, 341).

The mucus gel rests atop a thin fluid layer called the airway-surface liquid (ASL). The depth of the ASL is maintained through coordinated regulation of transcellular sodium absorption and anion secretion pathways within the epithelium, as well as paracellular ion and water fluxes (33, 145). The efficacy of mucociliary clearance depends on the integrity of these and other transport pathways to preserve the height of the ASL, thus facilitating effective ciliary beating (9, 357). Respiratory disorders such as cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD) are all associated with defective or ineffective mucociliary clearance that ultimately leads to chronic inflammation, airway remodeling and permanent loss of lung function (36, 44, 88, 103, 158, 238, 368).

Current treatment strategies for patients with asthma, COPD and other inflammatory airway disorders emphasize a combined therapy involving inhaled corticosteroid (ICS) and long-acting β_2-agonists (LABA) aimed at reducing inflammation and increasing airway caliber (17). LABAs, such as Salmeterol and Formoterol, are effective long-acting inhaled bronchodilators (27, 301, 358). These drugs stimulate increases in cAMP which activates protein kinase A (PKA) dependent signaling pathways in airway smooth muscle, leading to relaxation and a decrease in airway resistance (31). The therapeutic benefit of ICS treatment is attributed to transrepression of specific genes induced by proinflammatory transcription factors such as NF-kB and AP-1, limiting airway hyperresponsiveness (118, 170, 322, 391). Other effects of corticosteroids on airway epithelial cells suggest a role in surface cell diversity by increasing the number of ciliated epithelial cells and decreasing mucus hypersecretion, therefore promoting mucociliary clearance (79, 198).

Although effective at reducing inflammatory immune reactions and airway hyperresponsiveness, the effects of combined therapy on the airway epithelium are not as well characterized. Furthermore, hydrocortisone (HC) is one of the constituents of cell culture media that is commonly used for maintaining NHBE cells in culture. Therefore, the overall goal of the present study was to investigate the role of glucocorticoids in the development of the ion transport phenotype of bronchial epithelial cells during

differentiation. We hypothesized that hydrocortisone (HC) is necessary for *in vitro* differentiation of airway basal cells into a pseudostratified epithelium with ciliated and secretory surface cells. Additionally, we hypothesized a role for HC in the development of normal transepithelial ion-transport pathways essential for mucociliary clearance. The results of the present study demonstrate that early glucocorticoid exposure affects the development and maintenance of specific ion-transport pathways required for mucociliary clearance. These findings indicate that ICS treatment supports mucosal defense through direct interactions with the airway epithelium.

Materials and Methods

Materials

Retinoic acid, Benzamil hydrochloride, $CFTR_{inh}$-172, Salbutamol hemisulfate salt, 8-(4-Chlorophenylthio)adenosine 3′,5′-cyclic monophosphate sodium salt (8-CPT-cAMP), 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), and Uridine triphosphate (UTP) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Paraformaldehyde, 16% solution was purchased from VWR (Radnor, PA, USA).

Cell Culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Basel, CHE) and expanded in bronchial epithelial cell growth medium (BEBM + SingleQuots containing 1.4 □M hydrocor
low density on 0.4 μm pore-size Snapwell polyester membranes and maintained under liquid-liquid interface growth conditions with complete BEGM until cells reached confluency (day 0). Hydrocortisone (HC; 1.4μM) was withdrawn from HC0 and HC8 cells at day 0 and 8, respectively. Retinoic acid (RA; 500nM) was added to BEGM for 48hrs to promote differentiation (1). Air-liquid interface (ALI) culture conditions were initiated at day 2 by removing apical media and basolateral growth media was replaced with differentiation media (DMEM/F12 + SingleQuots) containing 100 nM RA. Cells

were harvested at four different time points: days 0, 4, 8 and 24. Transepithelial resistance (TER) measurements were made every-other day for 24 days before media change using "chopstick" electrodes and a voltohmmeter (EVOM: World Precision Instruments (WPI), New Haven, CT, USA). All cells were grown at 37^{o}C in a humidified CO_2 atmosphere.

Quantitative RT-PCR

RNA was isolated using the Rneasy Mini Kit from Qiagen (Hilden, DEU). cDNA was produced with the QuantiTect Reverse Transcription Kit with gDNA Wipeout also from Qiagen. Taqman PCR probes were purchased through Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR amplification of 5ng cDNA was performed on an Applied Biosystems 7300 Real-Time PCR system. Baseline and threshold values were set according to manufacturer's instructions. Relative expression was quantified using the $2^{-\Delta Ct}$ method with GAPDH as the reference gene (317).

Immunofluorescence and Western blot analysis

Cells were grown on Snapwell polyester membranes as described and fixed in 4% PFA for 20 min. Cell membranes were permeated using 0.3% Triton-X and blocked with a PBS + 3% BSA solution for at least 1 hr. Cells were incubated with the primary antibody overnight at 4°C diluted in 3% BSA solution. Following incubation, cells were rinsed three times in PBS and incubated with the secondary antibody for 1 hr at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Monolayers were excised and mounted on microscope slides using VECATSHIELD HardSet mounting media (Vector Laboratories, Burlingame, CA, USA). Images were captured using an Olympus FV1000 Confocal Microscope. Primary antibodies were purchased from Abcam (ENaCα, α1-NaKATPase, CFTR, β_2-AR, ZO1; Cambridge, GBR) or R&D Systems (Prss8/CAP1; Minneapolis, MN, USA) and secondary antibodies were purchased from Invitrogen (Alexa Fluor 488, 568, 647; Carlsbad, CA, USA).

Total protein was collected using Pierce IP Lysis Buffer and was quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 10 or 25ng total protein

was loaded on NuPAGE 4-12% Bis-Tris Gels with Chameleon Duo Pre-stained protein ladder (Li-Cor Biosciences, Lincoln, NE, USA) and separated using electrophoresis in MOPS SDS Running Buffer (200V, 50 min). Proteins were transferred onto activated Immobilon-FL PVDF membranes (Millipore Corporation, Billerica, MA, USA) and blocked in Odyssey Blocking Buffer overnight at 4°C (Li-Cor Biosciences). Primary antibodies were diluted in Odyssey Blocking Buffer containing 0.2% Tween-20 and incubated overnight, washed five times in PBS+0.1% Tween-20, and then incubated with IRDye secondary antibodies diluted in Odyssey Blocking Buffer for 40 mins (Li-Cor Biosciences). Blots were visualized with an Odyssey CLx Imager and analyzed using Image Studio Lite (Li-Cor Biosciences). β-actin primary antibodies were purchased from Santa Cruz Biotechnology (sc-69879/sc-130656, Dallas, TX, USA).

Electrophysiology

Short-circuit current (I_{SC}) measurements were performed on high-resistance monolayers (>700 $\mathbf{\Omega}\cdot cm^2$) mounted in Ussing chambers bathed on both sides with standard saline solution containing (in mM) 130 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 20 $NaHCO_3$, 0.3 Na H_2PO_4, and 1.3 Na_2HPO_4, pH 7.4, and maintained at 37°C with 95% O_2-5% CO_2 gassing.

Statistics

All values reported in the Results are expressed as the mean ± SEM. For the qRT-PCR experiments reported in figures 2, 6, 7, 8 and 9, an asterisk (*) represents significant differences between day zero and each subsequent day of differentiation as determined by an ANOVA followed by Dunnett's test for comparisons with a common control within each treatment condition. The open circles (o) represent significant differences between either the HC0 condition or the HC8 condition compared to the corresponding day of differentiation in the control group as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test.

In figures 4, 5 and 9 differences in blocker-sensitive or agonist-sensitive Isc are shown with either an asterisk (*) representing significant differences between HC0 and

control conditions or a circle (o) which represents a significant differences between HC0 and HC8 conditions as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test. A value of $p<0.05$ was considered significant.

Results

NHBE cells differentiate into a ciliated-pseudostratified epithelium

NHBE cells were expanded then differentiated on polyester Snapwell membranes according to the protocol described in the Methods. The cells were maintained under liquid-liquid conditions in complete BEGM growth media until they reached confluence (day 0). 1.4μM hydrocortisone (HC), supplied in the BEGM SingleQuots, was withdrawn from HC0 and HC8 cells at day 0 and 8, respectively. Apical media was withdrawn from control, HC0 and HC8 cells at day 2 and cells were maintained under ALI conditions for the duration of the experiment. Transepithelial resistance (TER) was measured every-other day as shown in Figure 1A. HC0 cells exhibited greater increases in TER at all days between 2 and 10 compared to control and HC8 cells. Under all three conditions, the mean TER value calculated using measurements from all of the days after day 8 exceeded 1000 $\Omega \cdot cm^2$ (mean TER ± SEM d8-d24 ($\Omega \cdot cm^2$): control = 1055 ± 85.9, HC0 = 1161 ± 79.8, HC8 = 1149 ± 93.5). Tight junction (TJ) formation was visualized by immunocytochemistry targeting the cytoskeletal scaffolding protein ZO1 (387). As shown in Figure 1B, ZO1 was localized at the lateral borders of control and HC0 cells at day 24, indicating mature TJ structures.

We examined changes in expression of specific mRNAs associated with differentiation of bronchial basal cells by qRT-PCR. The basal cell transcription factor p63 (Figure 2A) was expressed at the highest level in control cells at day 0, but exhibited a significant reduction in expression at days 4, 8 and 24. More p63 was detected in HC0 and HC8 cells at days 8 and 24 than the corresponding days in control monolayers indicating a role for HC in the expression of p63. Transcription of cytokeratin 6a (KRT6a: Figure 2B), a type II intermediate filament protein highly expressed in the airway basal cells (126), also showed HC dependent down-regulation in differentiated control cells. However, HC0 and HC8 cells expressed a greater amount of KRT6a

mRNA at day 24 than control (C_T = 25.4 ± 0.8, 24.3 ± 0.5, 28.7 ± 0.3, respectively). These results suggest that differentiation in the presence of HC reduced the abundance of basal cell markers within the epithelium but that basal cells are still present within differentiated, pseudostratified monolayers by day 24.

Next, the transcription of genes associated with airway surface cells was investigated. FoxJ1 (Figure 2C), a master transcription factor of ciliagenesis (407), as well as mucins Muc5ac (Figure 2D) and Muc5b (Figure 2E), were not detected at day 0 but were observed at days 4, 8 and 24 in control cells, indicating the presence of ciliated and secretory surface cells in differentiated monolayers. This was confirmed by immunocytochemistry (Figure 3A and B). HC0 and HC8 cells expressed higher levels of FoxJ1 mRNA at day 24 compared to control monolayers (C_T = 28.8 ± 0.5, 28.3 ± 0.5, 31.5 ± 0.3, respectively). Muc5ac was not detected in HC0 cells at day 24. Interestingly, transcription of Muc5b showed HC-dependent up-regulation in control cells, however it is worth noting that this does not necessarily indicate increased protein expression or mucus secretion in HC treated monolayers. HC withdrawal (HC8) cells exhibited reduced expression of Muc5ac and Muc5b at days 8 and 24 compared to control, similar to HC0 cells (Figure 2E). Moreover, detection of Muc5b by immunocytochemistry at the apical surface of day 24 differentiated HC0 monolayers was reduced compared to control and HC8 cells (Figure 3C and D). The secretoglobin protein Uteroglobin or Club Cell 10 protein (SCGB1A1, Figure 2F) also showed robust HC dependent expression during differentiation in the presence of HC, but significantly reduced expression at days 8 and 24 in HC0 cells and at day 24 in HC8 monolayers. This result suggested an increase in Club cell abundance within control monolayers during differentiation that required HC for sustained expression.

Hydrocortisone-sensitive sodium and anion transport in differentiated NHBE cells

Differentiated NHBE monolayers were mounted in Ussing chambers to investigate ion transport function by characterizing ENaC and CFTR dependent transport pathways. Day 8 control and HC8 cells had statistically greater total and benzamil-sensitive short circuit current (Isc) compared to HC0 monolayers, reflecting a greater

level of basal Na^+ absorption (Figure 4). HC had no effect on the magnitude of the $CFTR_{inh}$-172-sensitive Isc, indicating that the level of basal anion secretion was unaffected by the absence of hydrocortisone. Day 24 HC8 monolayers displayed a similar phenotype as HC0 cells (Figure 5). Total and benzamil-sensitive currents were significantly reduced in HC0 and HC8 cells compared to control.

Immunocytochemistry revealed the localization of ENaCα and the α_1-subunit of Na^+-K^+ ATPase at the apical and basolateral membranes, respectively, of differentiated NHBE monolayers (Figure 6A). ENaCα localized at cilia-like structures on surface cells of control monolayers. Surface ENaCα was reduced in HC0 cells. ENaCα was detected just below the apical membrane in the same focal plane as the Na-K ATPase α_1 subunit in HC0 and HC8 cells, implicating a role for HC in trafficking of ENaC to the apical surface and into the cilia through insertion of sub-apical membrane vesicles. Furthermore, qRT-PCR analysis of ENaCα and γ mRNAs displayed HC dependent increases at day 8 (Figure 6B and D). HC did not alter the localization of the Na^+-K^+ ATPase at the basolateral membrane as shown in Figure 6A. However, ATP1A1 mRNA was reduced in the absence of HC but showed no change in HC8 cells compared to control (Figure 6E), indicating that the initial 8 days of exposure to HC was sufficient to augment expression of the β_1 subunit.

Earlier studies have demonstrated that ENaCα and γ subunits are subject to cleavage by proteases that enable complete activation of the channel (152). Therefore, we examined whether glucocorticoid exposure was involved in regulating the expression of known proteases involved in ENaC processing. Our results showed an increase in mRNA expression of furin and channel-activating protease 1 (CAP1) during differentiation in control versus HC0 monolayers at days 8 and 24, although mRNA for both enzymes was still expressed at high levels in HC0 cells (Figure 6F and G). However, similar levels of the 37 kDa N-terminal cleavage product of ENaCα was observed in control and HC0 monolayers at days 8 and 24 (Figure 6H) (204). Serum and glucocorticoid-regulated kinase 1 (SGK1) mRNA, a known modulator of surface ENaC expression, was significantly reduced in HC0 monolayers at days 4 and 8 compared to control cells (Figure 6H). ATP1α1 protein was observed at days 8 and 24 in HC0 and

control cells (Figure 6H).

Anion secretion increases following β_2-AR stimulation

Signaling pathways coupled to ion transport in airway surface epithelial cells are integral to mucociliary clearance. We investigated the role of HC in development of normal β_2-adrenergic receptor regulation of CFTR-dependent anion secretion using the short-acting selective β_2 agonist salbutamol. Previous experiments (Figures 4 & 5) demonstrated that the transport phenotype of differentiated NHBE monolayers treated with HC was similar at day 8 and 24. Therefore, day 16 monolayers were pre-treated with benzamil before being stimulated with 10μM salbutamol. Treatment with $CFTR_{inh}$-172 after salbutamol stimulation inhibited the Isc in control, HC0 and HC8 monolayers (Figure 7A and B). HC0 and HC8 monolayers displayed less of an increase in Isc after addition of salbutamol compared to control monolayers (Figure 7B). qRT-PCR analysis of β_2-AR transcription (ADRB2) revealed HC dependent expression at days 8 and 24 (Figure 7C). CFTR mRNA expression showed a time-dependent decrease in HC0 cells while HC8 expression remained similar to control at day 24 (Figure 7D). Western blot analysis also revealed a time-dependent decrease in total CFTR abundance in HC0 cells. However, ADBR2 protein abundance did not appear to be reduced in the absence of HC. Immunocytochemistry results presented in Figure 8A and B showed co-localization of the β_2-AR and CFTR in cilia-like structures of control monolayers, but less distinct cilia localization in HC0 and HC8 monolayers.

To confirm that the salbutamol-activated Isc was dependent on functional CFTR expressed in the apical membrane, human Δ508 cystic fibrosis bronchial epithelial cells (CFBE) were differentiated according to the protocol used for control NHBE cells. CFBE monolayers displayed a reduced benzamil sensitive current compared to control (1.04±0.16 μA) and were unresponsive to salbutamol as well as 8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphate (8-CPT-cAMP), a membrane permeable analog of cAMP known to activate CFTR-dependent anion secretion (Figure 8C). As a positive control, CFBE cells were subsequently stimulated with a $P2Y_2$ receptor agonist (UTP) known to activate Ca^{2+}-dependent Cl secretion in airway epithelial cells (218). Although

CFTR-dependent anion secretion could not be activated by agents that increase cAMP, CFBE monolayers did exhibit UTP-dependent increases in Isc that could be blocked by DIDS, a disulfonic stilbene compound that inhibits Ca^{2+}-dependent Cl channels (240). CFBE monolayers also appeared to exhibit colocalization of the β_2-AR with CFTR (Figure 8D) although more extensive co-immunoprecipitation experiments would be needed to confirm this interpretation. However, CFTR expression was not localized in cilia-like structures of ΔF508 monolayers, appearing instead within the perinuclear region below the apical membrane.

Discussion

In vitro differentiation of NHBE cells under ALI conditions provides a unique opportunity to study signaling molecules that regulate the development of a physiologically responsive, pseudostratified epithelium. The technique has been employed by many groups to study the effects of cigarette smoke, pathogenesis of influenza infection, bronchial wound healing, and as a model for airway drug transport (78, 115, 209, 231, 346). The precise culture conditions used to differentiate bronchial basal cells into a monolayer containing secretory and ciliated cells are varied. Addition of retinoic acid to culture medium is a common practice which promotes pseudostratification and suppresses keratinizing squamous differentiation (58, 107, 239). Moreover, nearly all NHBE cell culture media contain glucocorticoids but the rationale for supplementation is poorly understood. Corticosteroids are well-studied modulators of multiple processes across a variety of tissues but their role in the differentiation of airway basal cells into a tissue capable of transepithelial salt and water transport has not been systematically investigated.

Detection of both ciliated and secretory cells at day 24 occurred in both HC0 and HC8 cells and did not appear to be different from control. However, HC did reduce transcription of mRNAs associated with secretory cells. Relative expression of Muc5ac, Muc5b and SCGB1A1 were all reduced in the absence of HC. Previous studies have reported suppression of goblet cell abundance in the presence of glucocorticoids (178, 290). Although not necessarily indicative of surface cell-type abundance, our data

suggests increased transcription of mucin proteins in the presence of HC. One possible explanation may involve HC dependent down-regulation of basal-cells occurring in parallel with an increase in surface cell expression as indicated by reductions in the levels of p63 and KRT6a and increased expression of surface cell markers. HC0 monolayers expressed greater relative amounts of p63 and KRT6a at later time-points than control cells which may be indicative of basal cell proliferation, potentially leading towards squamous metaplasia (8).

We also identified a role for HC in determining the magnitude of basal benzamil-sensitive Na^+ absorption in well-differentiated NHBE cells. Reduced sodium absorption was observed at day 8 and 24 in NHBE cells differentiated in the absence of HC (HC0). Furthermore, surface expression of ENaCα was reduced at day 24, although the α-subunit was detected by immunocytochemistry just below the apical membrane, suggesting localization within sub-apical membrane vesicles. In contrast, cells differentiated in the presence of HC exhibited ENaCα localization within cilia-like structures, consistent with previous results showing localization of ENaC within motile cilia of native airways as well as K2P channels in cilia of differentiated HBE cells (91, 412). Additionally, withdrawal of HC after eight days of differentiation (HC8) resulted in a similarly reduced benzamil-sensitive Isc at day 24, although unlike HC0 monolayers, ENaCα expression within the apical membrane was comparable to monolayers that were differentiated in the continuous presence of HC. One potential explanation for the reduced levels of Na^+ transport associated with HC0 monolayers is an effect of HC on mRNA expression of ENaC subunits. Glucocorticoids have been previously shown to directly regulate ENaC transcription and in this study, HC-dependent regulation of αENaC and γENaC, but not βENaC mRNA was observed when HC was removed at the start of the differentiation protocol. Earlier studies demonstrated dexamethasone/hydrocortisone-dependent up-regulation of all three ENaC subunits in human middle ear (177) and human mammary epithelial cells (38) respectively as well as dexamethasone but not aldosterone regulation of βENaC and γENaC mRNA expression in bovine mammary epithelial cells (287). Interestingly, dexamethasone did not stimulate an increase in ENaCα or γ protein

expression in CFBE41o$^-$ cells, an immortalized human bronchial epithelial cell line stably expressing wt CFTR or the ΔF508 CFTR mutation (304).

Furthermore, HC could also regulate ENaC function by altering the expression of enzymes involved in proteolytic processing of the channel. Previous investigations have shown that ENaCα and γ are subject to proteolytic cleavage, resulting in functionally mature sodium channels (152). Multiple proteases have been reported to mediate ENaC processing and we hypothesized a role of HC in regulating the expression of these enzymes based on previous reports showing that aldosterone directly controls the expression of prostasin, also known as channel-activating protease 1 (CAP1). Our results demonstrated that removal of HC prior to the start of differentiation reduced mRNA expression of two proteases known to regulate ENaC processing, including furin, which cleaves the extracellular loop of ENaCα and γ, and CAP1, which cleaves the extracellular loop of the γ subunit (40). Although we observed a reduction in ENaCα mRNA in the absence of HC, we detected similar total abundance of cleaved ENaCα by western blot, indicating a mechanism other than transcription is responsible for the reduced benzamil-sensitive current observed in HC0 and HC8 monolayers.

The observation that HC0 monolayers exhibited reduced ENaC protein expression within the apical membrane supports a role for HC in the trafficking of channel subunits to the apical surface. ENaC retrieval from the plasma membrane is known to be regulated by the ubiquitin-protein ligase, Nedd4-2. Nedd4-2 activity is itself controlled by serum/glucocorticoid-regulated kinase 1 (SGK1). SGK1-mediated phosphorylation of Nedd4-2 reduces its interaction with ENaC leading to an increase in channel density in the apical membrane (80, 171). Therefore, in this study we tested the hypothesis that removal of HC from the media at the start of differentiation reduces SGK1 mRNA expression, which in turn reduces ENaC surface density. SGK1 mRNA expression was reduced in the absence of HC compared to control and HC8 monolayers, consistent with previous studies on HC stimulation of ENaC surface expression through regulation of the SGK1-Nedd4-2-ENaC axis in middle ear epithelial cells (177).

In addition to regulation of ENaC function, the mineralocorticoid hormone aldosterone has been shown to directly stimulate Na^+-K^+ ATPase activity and

transcription in distal nephron and collecting duct epithelial cells resulting in increased sodium absorption and water retention (311). Similarly, dexamethasone has been shown to regulate Na^+-K^+ ATPase activity in corneal endothelial cells and to increase transcription of the β_1 subunit of the enzyme in renal carcinoma cells (134, 154). Therefore we investigated whether HC was involved in regulating ATP1A1 localization and expression during differentiation of NHBE cells. Our results demonstrated reduced transcription of ATP1A1 mRNA in the absence of HC but no effect on membrane localization of the protein. The reduced expression and protein abundance of the α_1 subunit of the pump was consistent with the decrease in ENaC mRNA and surface expression of ENaCα subunits in HC0 monolayers and may have contributed to the lower rate of basal Na^+ transport. Interestingly, once differentiation was initiated and HC exposure sustained for 8 days (HC8 monolayers), withdrawal of HC for the next 16 days did not reduce ATP1A1 mRNA expression. A similar effect was observed for ENaCα, β and γ mRNA expression in HC8 monolayers, supporting continuous expression of ENaC subunits within the apical membrane at day 24. Transcriptional regulation by HC involves histone modifications which may have effects lasting longer than the time period of HC exposure and may explain sustained gene transcription of ENaC and ATP1A1 after HC withdrawal (16). Curiously, despite the sustained expression of ENaC and ATP1A1 mRNA subunits and their appropriate membrane localization, basal Na^+ transport in HC8 monolayers at day 24 was reduced to a similar level as observed in HC0 monolayers. At this time the mechanistic basis for the reduction in basal ENaC-dependent Isc is unknown, but one possible explanation could be a decrease in basolateral K^+ conductance, which would reduce the driving force for transepithelial Na^+ absorption.

Previous studies have suggested that ICS potentiates LABA efficacy by enhancing the functional response to β_2-AR agonists (2, 259). In the present study, qRT-PCR results indicated that HC plays a role in regulating the transcription of CFTR mRNA. Although the basal $CFTR_{inh}$-172-sensitive Isc was not affected by HC, salbutamol stimulated currents did show differences between control and HC-deficient conditions measured at day 16. The increase in Isc following addition of salbutamol was blocked by the addition of $CFTR_{inh}$-172 and was not observed in ΔF508 CFTR

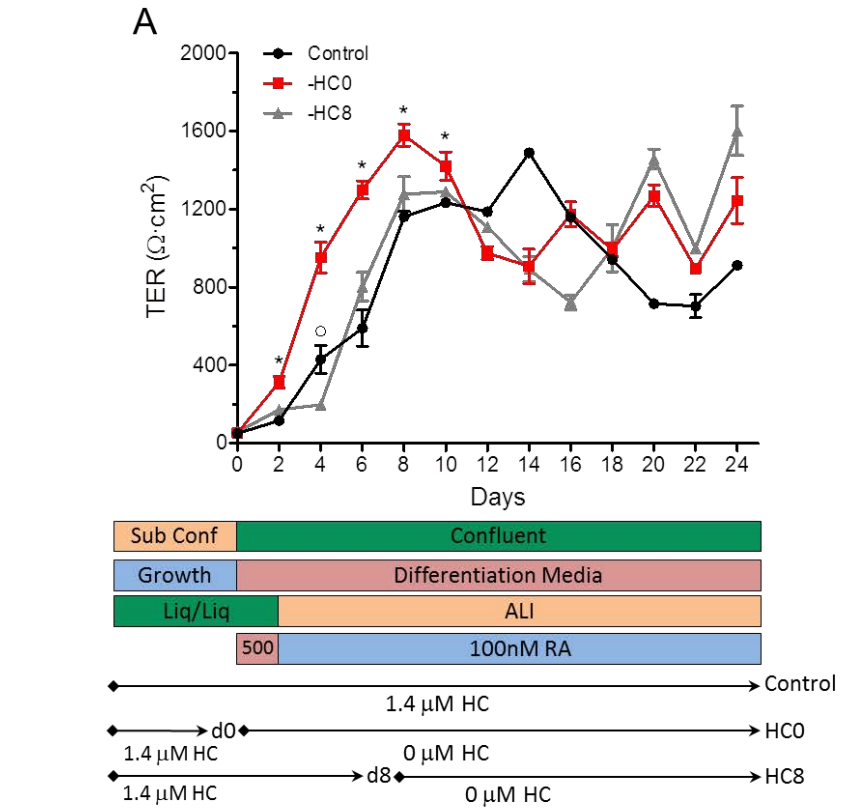
expressing monolayers which lacked functional CFTR. Earlier studies have shown that CFTR forms a signaling complex with β_2-ARs and here we show co-localization of CFTR and β_2-AR in NHBE cells and in ΔF508 CFTR expressing monolayers. Although this protein-protein interaction between CFTR and the β_2-AR appeared to show no dependence on HC, the relative abundance of β_2-AR mRNA was HC-dependent such that in the absence of HC, mRNA transcription was significantly reduced, although β_2-AR protein expression was sustained at levels comparable to control conditions. Glucocorticoid effects on β_2-receptors in airway epithelial cells and other tissues have been previously reported (2, 56, 62). These studies suggested a role for glucocorticoids as a permissive hormone in regulating the expression and density of β_2-ARs in plasma membranes, including airway smooth muscle cells (127, 222). Furthermore, glucocorticoids have been reported to protect airway smooth muscle from pro-asthmatic effects of LABAs through indirect suppression of phosphodiesterase E4 (259). Although basal CFTR currents were not different between control and HC0 conditions, salbutamol-induced CFTR currents in HC0 and HC8 cells were significantly lower than control monolayers. The reduced Isc response in HC0 cells may have been due to the decrease in β_2-AR expression, however mRNA levels in HC8 cells were comparable to control monolayers, so other mechanisms are likely to be involved in these cells.

Summary and Conclusions

The key findings of this study that relate to the effects of glucocorticoids during differentiation include i) enhanced mRNA expression of ENaCα, β and γ subunits, ii) coordinated up-regulation of ENaC processing (Furin/CAP1) enzymes as well as SGK1, iii) increased expression of CFTR and β_2 adrenergic receptors, iv) colocalization of ENaC, CFTR and β_2 adrenergic receptors within the cilia of surface cells, v) increased Na-K ATPase α_1 subunit expression and vi) increased CFTR-dependent anion secretion evoked by stimulation with β_2 adrenergic receptor agonists. These results support the conclusion that glucocorticoid treatment facilitates normal differentiation and development of ion transport protein expression and function critical for mucociliary clearance. Furthermore, glucocorticoid exposure during differentiation appears to be

necessary for the expression of apical β_2-ARs that regulate CFTR-dependent anion secretion in response to inhaled β_2-agonists. Thus the combined clinical use of glucocorticoids and LABAs for the treatment of asthma and other inflammatory airway diseases would be expected to promote epithelial restitution and mucociliary clearance necessary for maintaining mucosal barrier function and innate defense against airway pathogens. Inclusion of glucocorticoids in media used for differentiating airway progenitor cells may also be necessary for producing a fully functional pseudostratified airway epithelium for use in the bioengineering of human airways and perhaps ultimately, intact human lungs.

Figure 2.1


A
Control
-HC0
-HC8
TER (Ω·cm²)
2000
1600
1200
800
400
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Days
Sub Conf
Confluent
Growth
Differentiation Media
Liq/Liq
ALI
500
100nM RA
Control
1.4 µM HC
d0
HC0
1.4 µM HC
0 µM HC
d8
HC8
1.4 µM HC
0 µM HC

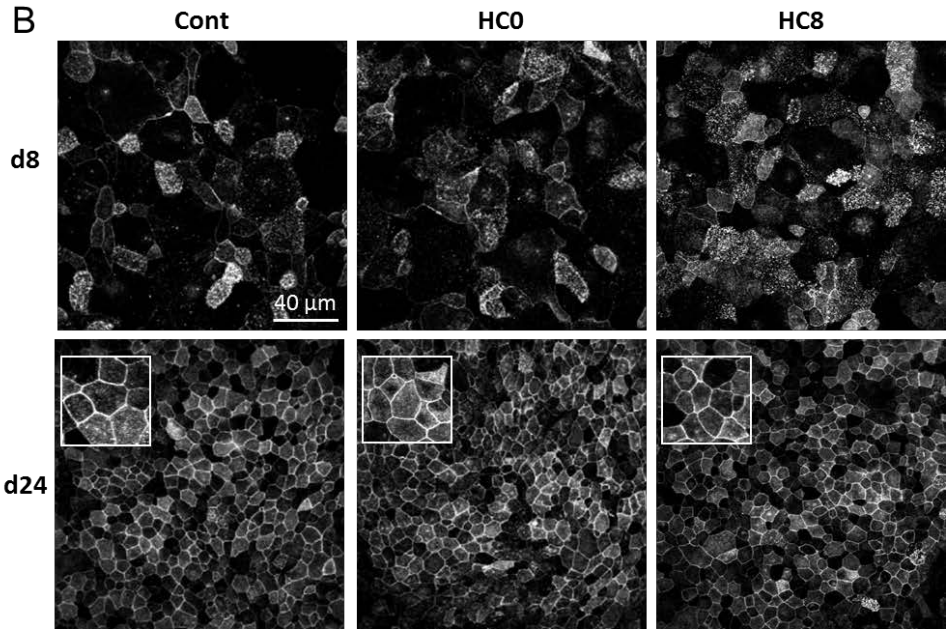

B
Cont
HC0
HC8
d8
d24
40 µm

Figure 2.1. NHBE cells develop tight junctions and form electrically tight monolayers during differentiation.

A. NHBE cells grown under standard differentiation conditions develop and maintain transepithelial resistances (TERs) over a 24 day period. HC0 cell TERs exhibited greater increases at earlier time points compared to control and HC8 cells. The asterisk (*) represents significant differences between the HC0 condition and Control/HC8 conditions, whereas open circles indicate a significant difference between Control and HC8 conditions as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test (n = at least 6 for each time point). Differentiation conditions are graphically represented below the graph. **B.** Localization of ZO1 immunofluorescence along the basolateral membrane in control, HC0 and HC8 cells. Inset panels are 30μm x 30μm images of day 24 monolayers.

Figure 2.2

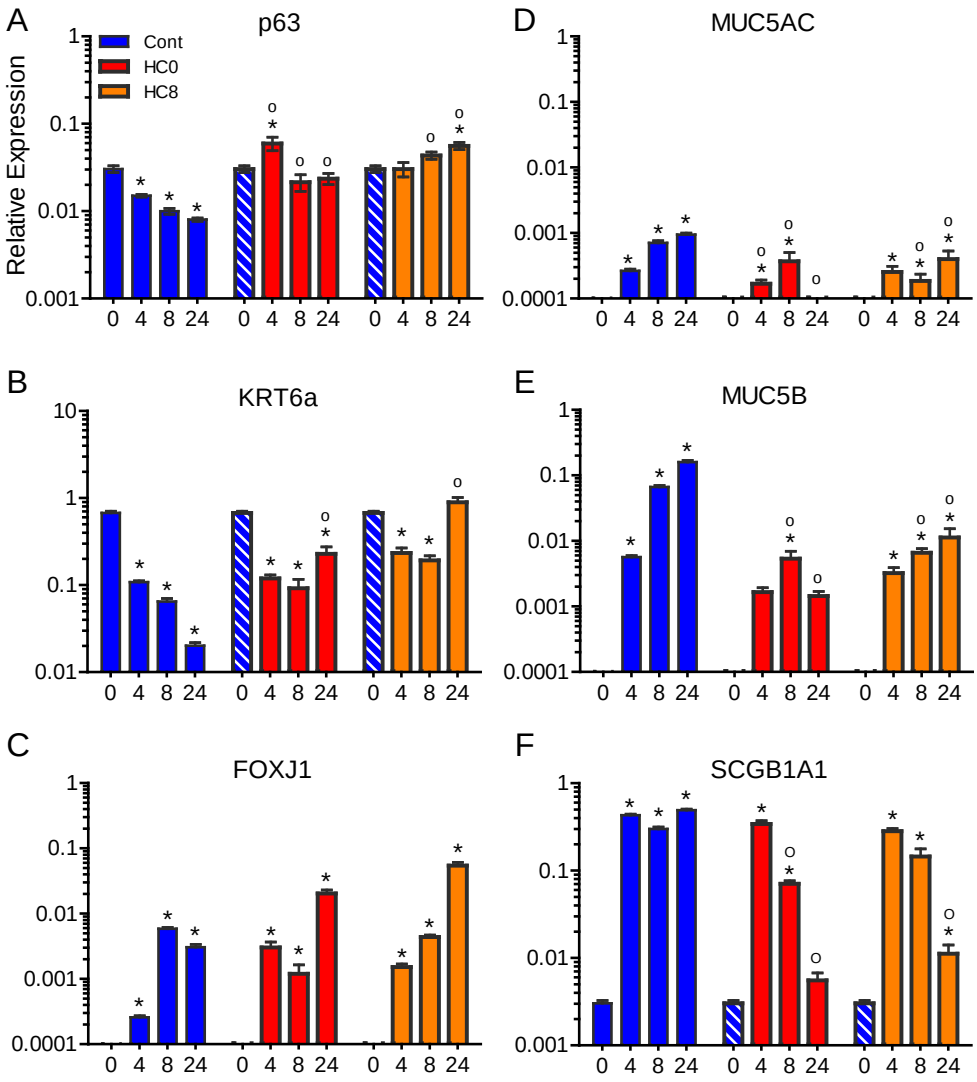

A
p63
Cont
HC0
HC8
Relative Expression
B
KRT6a
C
FOXJ1
D
MUC5AC
E
MUC5B
F
SCGB1A1
0 4 8 24

Figure 2.2. Differentiated NHBE cells express mRNAs associated with bronchial basal cells and surface cells.

A-C. qRT-PCR analysis showing mRNA expression of consensus bronchial basal cell markers p63, cytokeratin 6a (KRT6a) and FoxJ1 in undifferentiated NHBE cells (day 0) and differentiated NHBE monolayers (n = 6 for each condition). Data was normalized to GapDH and C_T values were 23.1±0.19, 23.1±0.56, 23.7±0.28 for control, HC0, and HC8 d24 respectively. Blue and white striped bars represent undifferentiated control data at day 0 (n = 6 for each bar). **D-F.** mRNA expression of bronchial surface cell markers were not detected or expressed at low levels in undifferentiated NHBE cells. However, by day 4, Muc5ac, Muc5b and Scgb1a1 were detected (n = 6 for each bar).

Figure 2.3

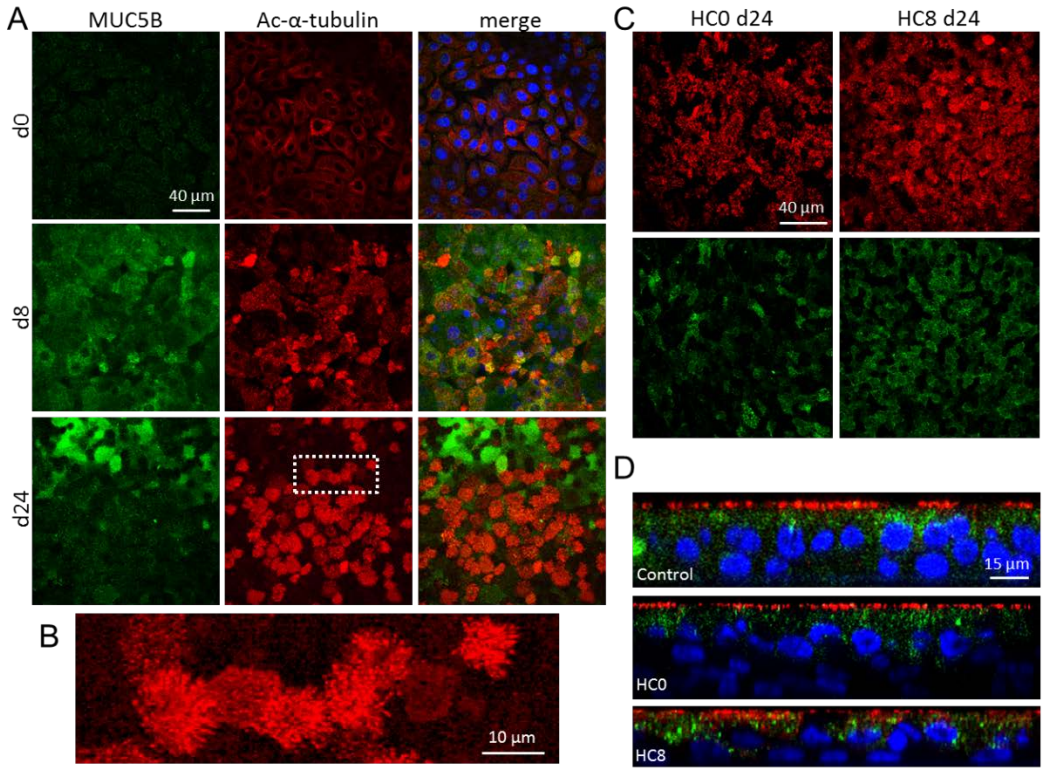


Figure 2.3. Immunocytochemistry of differentiated NHBE cells reveals a pseudostratified epithelium with ciliated and mucin-containing cells.

A. NHBE control monolayers contained surface cells expressing Muc5b (green) and acetylated α-tubulin (red), characteristic of cilia during differentiation. Nuclei (blue) were labeled with DAPI. **B.** Enlarged image identified in the white box of day 24 acetylated α-tubulin labeled cilia (in part A) on the apical surface of differentiated monolayers. **C.** HC0 and HC8 monolayers contained both ciliated and Muc5b-expressing cells. **D.** Orthogonal views of differentiated cells showed pseudostratification and apical localization of acetylated α-tubulin.

Figure 2.4

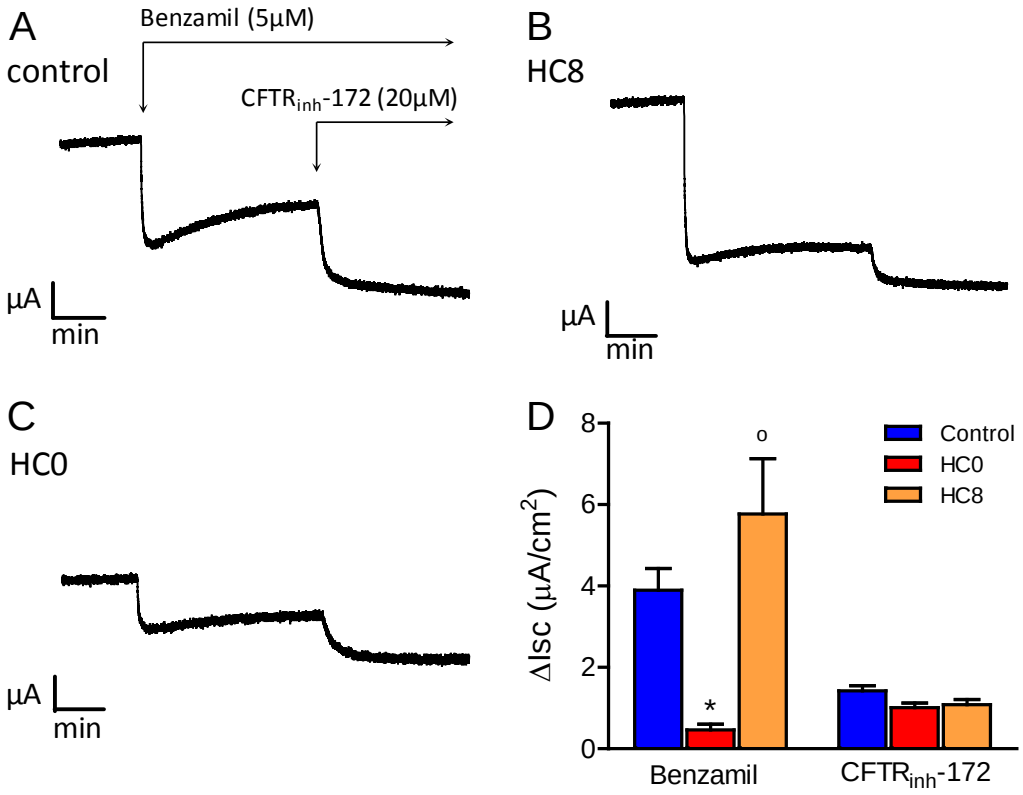


Figure 2.4. Day 8 HC0 cells have reduced benzamil-sensitive current but normal $CFTR_{inh}$-172-sensitive current compared to hydrocortisone treated controls.

A-C. Representative I_{SC} traces of day 8 differentiated monolayers treated with 5 µM benzamil, a selective blocker of epithelial sodium channels (ENaC), and 20 µM $CFTR_{inh}$-172, a selective CFTR blocker. **D.** Histogram summarizing I_{SC} results reported in A-C (n = 6 for each condition).

Figure 2.5

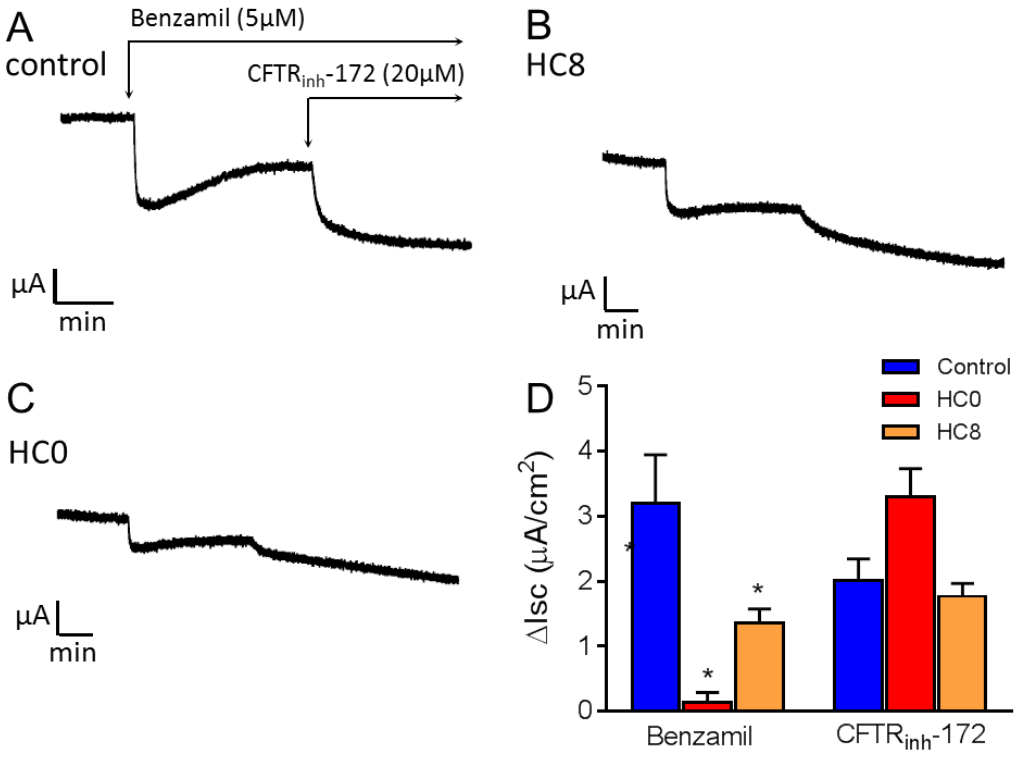


Figure 2.5. Day 24 HC0 and HC8 cells have reduced total and benzamil-sensitive currents compared to control.

A-C. Representative I_{SC} traces of day 24 differentiated monolayers treated with 5 μM benzamil and 20 μM $CFTR_{inh}$-172. **D.** Histogram summarizing the I_{SC} results reported in A-C (n = 6 for each condition).

Figure 2.6

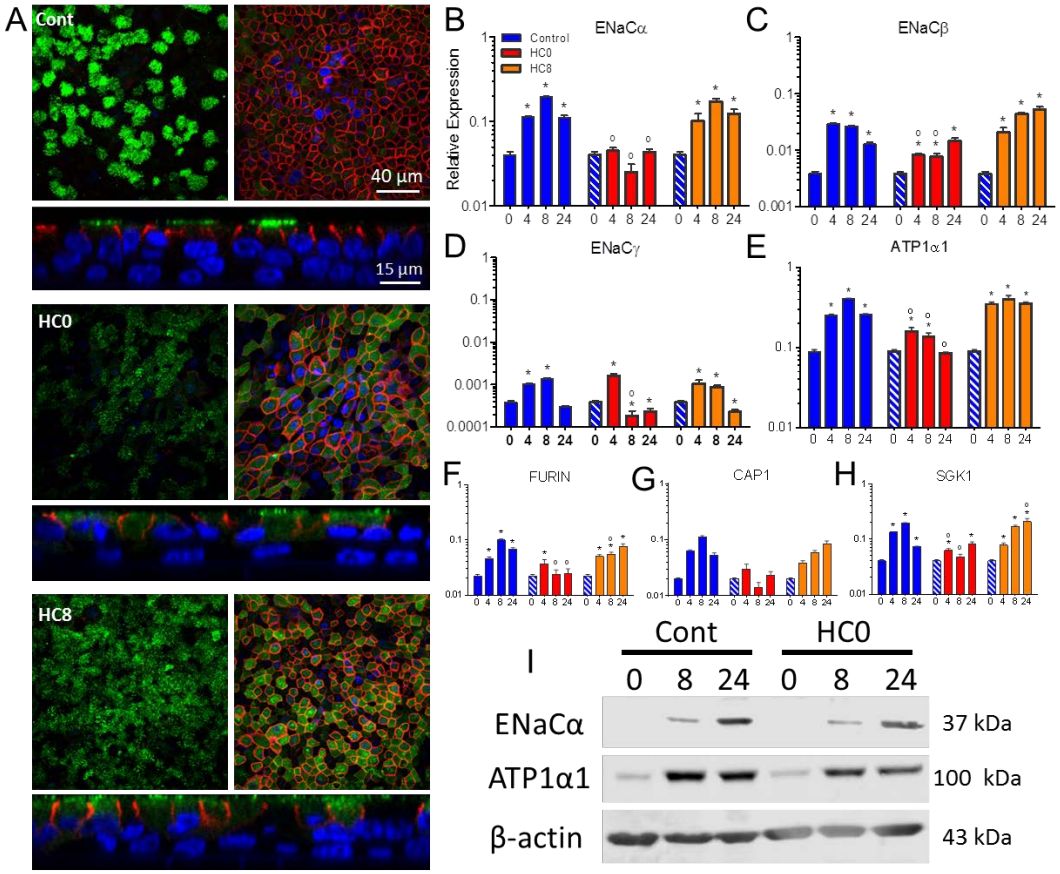

A
Cont
40 µm
15 µm
HC0
HC8
B
ENaCα
Control
HC0
HC8
Relative Expression
C
ENaCβ
D
ENaCγ
E
ATP1α1
F
FURIN
G
CAP1
H
SGK1
I
Cont
HC0
0 8 24 0 8 24
ENaCα
ATP1α1
β-actin
37 kDa
100 kDa
43 kDa

Figure 2.6. HC0 cells express lower ENaC mRNA levels and have less apically localized ENaCα subunits compared to control and HC8 cells.

A. Immunocytochemistry comparing control, HC0 and HC8 monolayers at day 24 showing localization of ENaCα (green) and Na^+-K^+ ATPase α_1 subunit (red) at the apical and basolateral membranes, respectively. Left panels show detection of ENaCα at the apical membrane. Right panels show detection of Na^+-K^+ ATPase α1 along the lateral boarder just beneath the apical membrane of the same monolayer. Nuclei (blue) were labeled with DAPI. **B-E.** qRT-PCR analysis showing the relative expression of ENaCα, β, and γ subunits as well as ATP1A1mRNAs (n = 6 for each bar). **F-H.** qRT-PCR analysis of mRNAs associated with ENaC proteolytic processing (FURIN, CAP1) and trafficking (SGK1) (n = 6 for each bar). **I.** Western blot analysis of cleaved ENaCα and ATP1α1 in control and HC0 cells (10 ng total protein). The ENaCα antibody recognizes a transmembrane region downstream of the amino-terminus of full length ENaCα.

Figure 2.7

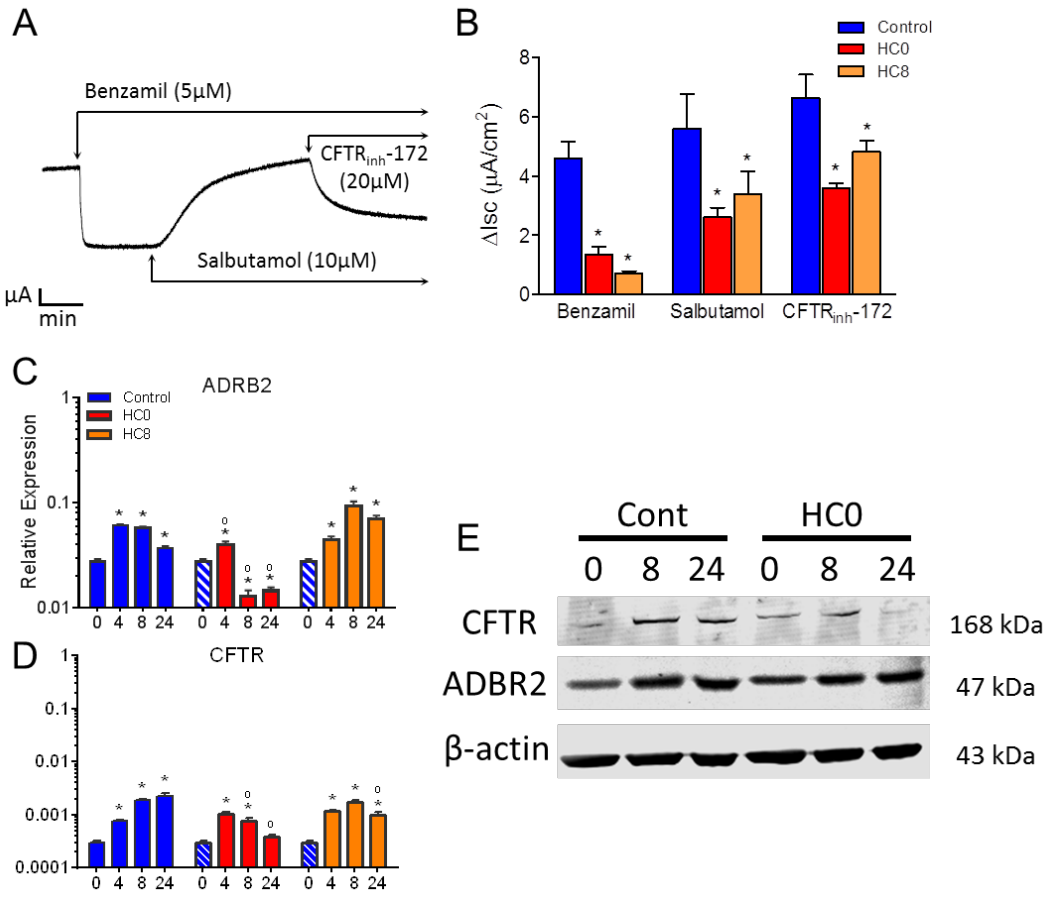

A
Benzamil (5µM)
CFTRinh-172 (20µM)
Salbutamol (10µM)
µA
min
B
Control
HC0
HC8
ΔIsc (µA/cm2)
Benzamil
Salbutamol
CFTRinh-172
C
ADRB2
Relative Expression
D
CFTR
E
Cont
HC0
0 8 24 0 8 24
CFTR
ADBR2
β-actin
168 kDa
47 kDa
43 kDa

Figure 2.7. Differentiated NHBE monolayers display increased I_{sc} responses after apical treatment with the selective β_2-AR agonist salbutamol.

A. Representative I_{SC} trace of day 16 monolayers treated apically with benzamil (5 μM) then the selective β_2-AR agonist salbutamol (10 μM), then $CFTR_{inh}$-172 (20μM). Apical addition of salbutamol increased the I_{sc} in both control HC0 and HC8 monolayers, consistent with stimulated anion secretion. **B.** Histogram summarizing benzamil sensitive, salbutamol activated and CFTR-dependent I_{SC} results reported in A. An asterisk (*) represents significant differences between treatment and control conditions (n = 4). **C-D.** qRT-PCR analysis of β_2-AR (ADRB2) and CFTR mRNAs (n = 6 for each bar). HC0 monolayers showed reduced ADRB2 and CFTR expression at days 8 and 24. **E.** Western blot analysis of CFTR and ADBR2 in control and HC0 cells (25 ng total protein). CFTR protein abundance appeared to be reduced in HC0 cells at days 8 and 24.

Figure 2.8

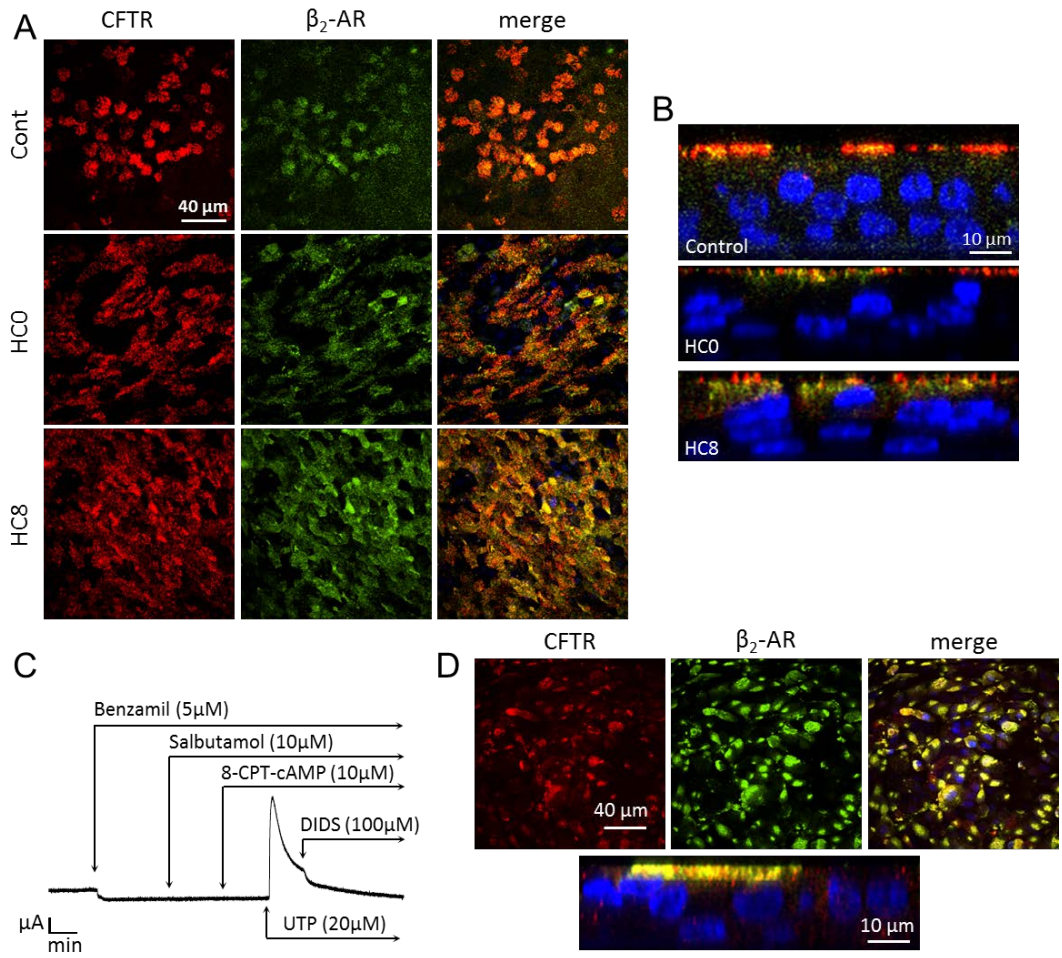

A
CFTR
β2-AR
merge
Cont
HC0
HC8
40 µm
B
Control
HC0
HC8
10 µm
C
Benzamil (5µM)
Salbutamol (10µM)
8-CPT-cAMP (10µM)
DIDS (100µM)
UTP (20µM)
µA
min
D
CFTR
β2-AR
merge
40 µm
10 µm

Figure 2.8. CFTR and β_2-AR co-localize at the apical membrane of differentiated NHBE monolayers.

A. Immunocytochemistry shows co-localization of CFTR (red) and β_2-AR (green) at the apical membrane in day 24 differentiated control, HC0 and HC8 monolayers. Co-localization of CFTR and β_2-AR was detected in cilia-like structures. Nuclei (blue) were labeled with DAPI. **B.** Orthogonal views of day 24 differentiated cells showing apical localization of CFTR and β_2-ARs within cilia. **C.** Representative I_{SC} trace of day 16 ΔF508 CFBE monolayers treated apically with benzamil (5 μM) then the selective β_2-AR agonist salbutamol (10 μM). 8-CPT-cAMP (10 μM) was then added to the apical and basolateral compartments but no change in I_{sc} was detected. Stimulation with UTP (20 μM) produced an increase in Isc that was blocked by the disulfonic stilbene derivative DIDS (100 μM), a Ca^{2+}-activated Cl channel blocker. **D.** Immunocytochemistry shows localization of CFTR (red) and β_2-AR (green) in the perinuclear region below the apical membrane in differentiated ΔF508 CFBE monolayers. Localization of CFTR and β_2-AR was not detected in cilia-like structures. Nuclei (blue) were labeled with DAPI.

Chapter 3

Large Conductance Ca^{2+}-activated K^+ Channel Activation by Purinergic Receptor Agonists Requires Hydrocortisone in Differentiated Normal Human Bronchial Epithelial Cells.

Nathan A. Zaidman[†], Angela Panoskaltsis-Mortari[†*] and Scott M. O'Grady[†§]

Departments of Integrative Biology and Physiology[†], Pediatrics[*], and Animal Science[§], University of Minnesota, 6-125 Jackson Hall, Minneapolis MN.

Synopsis

In the present study we investigated the role of hydrocortisone (HC) on UTP-stimulated ion transport in differentiated, pseudostratified epithelia derived from normal human bronchial basal cells. The presence of a UTP stimulated, paxilline-sensitive BK current was demonstrated in control epithelia but was not stimulated in cells that were differentiated in the absence of HC (HC0). Addition of the BK opener NS11021 activated channels in control epithelia as well as HC0, though only in the presence of UTP. The pan-specific PKC inhibitors GF109203x and Gö6983 blocked BK activation by UTP in control epithelia, suggesting PKC-mediated phosphorylation plays a permissive role in purinoceptor-stimulated BK activation. HC0 epithelia were found to express significantly more KCNMA1 containing the stress-regulated exon (STREX), a splice-variant of the α-subunit that displays altered channel regulation by phosphorylation. Control epithelia did not express significant amounts of STREX containing KCNMA1. Furthermore, BK channels as well as purinergic receptors were shown to localize in unique and overlapping membrane domains at the apical membrane of differentiated surface cells. The results presented here establish a previously unrecognized role of glucocorticoids in purinergic regulation of BK channels in airway epithelial cells.

Introduction

An important component of airway host-defense involves the process of trapping and removing inspired material through mucociliary clearance (MCC) (305). Mucins secreted by airway goblet cells form a viscous hydrogel that rests on the airway surface liquid (ASL). Regulated transepithelial ion transport supports this process by maintaining the depth of the ASL. This is accomplished by coordinating sodium absorption, mediated by apical epithelial sodium channels (ENaC) with anion secretion to maintain surface hydration. The importance of salt and water transport in MCC is emphasized in cystic fibrosis, where the absence of chloride secretion due to mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) and enhanced ENaC-dependent Na^+ absorption reduces the depth of the ASL leading to mucus stasis and chronic respiratory infection (381). Moreover, recent studies have revealed the contribution of large-conductance calcium-activated K^+ channels ($K_{Ca}1.1$, BK) to the maintenance of ASL depth (223).

BK channels are ubiquitously expressed in many different tissues and are critical to numerous physiologic processes, including regulation of vascular tone, neuronal excitability, synaptic transmission and hormone secretion (41, 87, 308, 389, 393). Allosteric activation of BK occurs in response to elevated intracellular [Ca^{2+}] as well as membrane depolarization, the only K^+ channel that possess both characteristics. BK channels are composed of a homotetramer of α-subunits combined with auxiliary β- and γ-subunits that are expressed in a tissue-dependent manner (183, 328). These auxiliary subunits tune the biophysical properties of BK channels by modifying Ca^{2+} and voltage sensitivity, permitting diverse biologic utility of BK's large single channel conductance (100-300pS) (195). For example, γ-subunits belonging to the leucine-rich repeat-containing (LRRC) protein family are expressed in non-excitable cells and shift the voltage-dependent activation of the channel to less depolarized potentials (~-140mV) (398).

Additionally, BK subunits are subjected to numerous post-transcriptional and post-translation modifications that further modify single channel activity (332). Phosphorylation of the α-subunit by various protein kinases has been well studied in a

variety of tissues. In vascular smooth muscle and in neurons, PKA and PKG mediated phosphorylation synergistically stimulate BK activity (196). In contrast, phosphorylation by PKC was shown to inhibit PKA and PKG mediated channel regulation in smooth muscle cells that express an α-subunit splice variant containing the dynamic and well-studied stress-regulated exon (STREX) (414, 415). However, PKC-dependent phosphorylation also stimulates channel activity as demonstrated by transfection of HEK293 cells with BK α-subunits containing a phospho-null mutation at S1076, which attenuated phorbol ester-induced BK activation (418). The interplay of differential phosphorylation at multiple sites by multiple kinases underscores a complex regulatory network employed by cells to limit unwarranted BK activation.

BK activity in airway epithelial cells is required for proper MCC. Knockdown of BKα (KCNMA1) transcripts, or constitutive channel inhibition was shown to decrease ASL volume and reduce ciliary beat frequency in freshly isolated bronchial epithelial cells (223). Apical K^+ secretion mediated by BK sustains Cl^- efflux following purinergic receptor stimulation and provides additional solute to increase the osmotic gradient that facilitates airway hydration. Furthermore, mediators of airway inflammation, like IFN-γ and TGF-β, suppress BK activity and deplete ASL volume, highlighting the important role of BK under normal physiological conditions (224, 225). These results also align with the reduced or impaired MCC observed in many airway diseases, such as CF, asthma, and COPD (30, 35, 39, 165, 212).

Our recently published results demonstrated the influence of glucocorticoids (hydrocortisone (HC)) on the establishment and maintenance of ion transport properties of human bronchial epithelium during mucociliary differentiation (408). Normal human bronchial epithelial (NHBE) cells differentiated in the absence of HC displayed significantly less benzamil-sensitive Na^+ absorption and a reduced short circuit current (I_{SC}) response after stimulation with the selective β_2-adrenergic receptor agonist salbutamol. In the present study, we investigated the effects of HC on purinergic receptor stimulated ion transport in differentiated bronchial epithelial cells. The results of this study established a significant and necessary role for glucocorticoids in the regulation of

BK channel function within the apical membrane during differentiation and highlight a previously unidentified role for glucocorticoid treatment in MCC.

Materials and Methods

Materials – Retinoic acid (RA), benzamil hydrochloride, CFTRinh-172, phorbol 12-myristate 13-acetate (PMA), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate (DIDS), adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATP-γ-S), adenosine triphosphate (ATP) and uridine triphosphate (UTP) were purchased from Sigma-Aldrich Chemical (St. Louis, MO). NS11021, CaCCinh-AO1, GF109203x, and Gö6983 were purchased from Tocris (Bristol, UK). Paraformaldehyde, 16% solution, was purchased from VWR (Radnor, PA). Normal human bronchial epithelial (NHBE) cells, BEGM and additional SingleQuot media supplement kits were purchased from Lonza (Basel, Switzerland).

Cell culture – NHBE cells were grown and differentiated as previously reported (408). Briefly, cells were expanded in bronchial epithelial cell growth medium [BEGM + SingleQuots containing 1.4 μM HC] and plated at low density on Snapwell polyester membranes and maintained under liquid-liquid interface growth conditions with complete BEGM until cells reached confluence (day 0). HC (1.4 μM) was withdrawn from HC0 cells at day 0. RA (500 nM) was added to BEGM for 48 h to promote differentiation. Air-liquid interface (ALI) culture was initiated at day 2 by removal of apical medium, and basolateral growth medium was replaced with differentiation medium (DMEM/Ham's F12 + SingleQuots) containing 100 nM RA. All cells were grown at 37°C in a humidified CO_2 atmosphere.

Quantitative RT-PCR – RNA was isolated from differentiated NHBE cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed as previously reported (408). Briefly, cDNA was produced with the QuantiTect reverse transcription kit containing gDNA Wipeout (Qiagen). TaqMan PCR probes were purchased from Life Technologies (Thermo Fisher Scientific, Waltham, MA). Specific TaqMan assays were: KCNMA1 WT (Hs01119498_m1), STREXi (Hs01119493_m1), STREXii (Hs01119490_m1). Quantitative PCR amplification of 5 ng cDNA was performed on a

real-time PCR system (model 7300, Applied Biosystems). Baseline and threshold values were set according to manufacturer's instructions. Relative expression was quantified using the comparative threshold cycle ($2^{-\Delta Ct}$) method. GAPDH expression was used as the reference gene.

Immunofluorescence and Western blot analysis – NHBE cells grown on Snapwell polyester membranes were fixed in 4% paraformaldehyde for 20 min, as previously described (408). Cell membranes were permeated with 0.3% Triton X-100 and then blocked with a 3% BSA solution for ≥1 h. Cells were incubated overnight at 4°C with the primary antibody diluted in BSA solution. After incubation, cells were rinsed three times in PBS and incubated with the secondary antibody for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Monolayers were excised and mounted on microscope slides with VECATSHIELD HardSet mounting medium (Vector Laboratories, Burlingame, CA). Images were captured from an Olympus FV1000 confocal microscope. The primary antibodies targeting maxi-K^+ channel alpha ($K_{Ca}1.1$, BKα) (ab99046), epithelial Na^+ channel α-subunit (ENaCα) (ab65710), acetylated α-tubulin (ab24610), $P2Y_4$ receptor (ab140857), and TMEM16a (ab53212) were purchased from Abcam (Cambridge, UK) and $P2Y_2$ receptor (sc-15209) was purchased from Santa Cruz Biotechnology (Dallas, TX); the secondary antibodies Alexa Fluor 488, 568, and 647 were purchased from Invitrogen (Carlsbad, CA).

Total NHBE protein was collected using Pierce immunoprecipitation lysis buffer and quantified with the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Ten or 25ng of total protein were loaded on NuPAGE 4–12% Bis-Tris gels with Chameleon Duo-prestained protein ladder (Li-Cor Biosciences, Lincoln, NE) and separated using electrophoresis in MOPS SDS buffer (200 V, 50 min). Proteins were transferred to activated Immobilon-FL polyvinylidene difluoride membranes (Millipore, Billerica, MA) and blocked in Odyssey blocking buffer overnight at 4°C (Li-Cor Biosciences). Primary antibodies were diluted in Odyssey blocking buffer containing 0.2% Tween 20 and incubated for 1 hour at room temperature, washed five times in PBS + 0.1% Tween 20, and then incubated with IRDye secondary antibodies diluted in Odyssey blocking buffer for 40 min. Blots were visualized with an Odyssey CLx imager

and analyzed using Image Studio Lite (Li-Cor Biosciences). Primary antibodies targeting PKCα (ab32376), PKCδ (ab182126), PKCε (ab114020), BKβ3 (ab137041), and BKβ4 (ab105587) were purchased from Lonza. LRRC26 (sc-102015) and β-Actin (sc-69879) primary antibodies were purchased from Santa Cruz Biotechnology.

Electrophysiology - Short-circuit current (I_{SC}) was measured on high-resistance (>700 Ω·cm2) monolayers mounted in Ussing chambers. NHBE cells were bathed on both sides with standard saline solution containing (in mM) 130 NaCl, 6 KCl, 1.5 CaCl2, 1 MgCl2, 20 NaHCO3, 0.3 NaH2PO4, and 1.3 Na2HPO4, pH 7.4, and maintained at 37°C with 95% O2-5% CO2.

Intracellular [Ca^{2+}] measurements – Differentiated NHBE cells were washed with HBSS containing 10 mM HEPES buffer, pH 7.4. Cells were loaded with Fura-2-AM for 1 hour then washed in HBSS and mounted onto the stage of a Nikon Diaphot inverted microscope. Single cell fluorescence was visualized using a Nikon UV-fluor 20x objective. Excitation (340 nm/380 nm), image acquisition and data analyses were performed using Image-1 MetaMorph software. $[Ca^{2+}]_i$ was measured as the ratio of fluorescence emitted at 510 nm when the cells were alternately excited at 340 nm and 380 nm (F340/F380).

Statistics – For quantitative RT-PCR experiments (Figs. 2, 3, 5 and 6) and ΔI_{SC} measurements in Figure 1, significant differences between day 8 differentiated epithelia were determined using Student's t-test. In Figures 3, 4 and 5, significant differences between ΔI_{SC} measurements were determined by ANOVA followed by a Tukey-Kramer multiple-comparisons test. $p < 0.05$ was considered significant.

Results

Paxilline-sensitive I_{SC} is HC dependent in differentiated NHBE cells.

NHBE cells were expanded then differentiated on polyester Snapwell membranes according to the protocol described in the *Methods*. The cells were maintained under liquid-liquid conditions in complete BEGM growth media containing 1.4 μM HC until they reached confluence (day 0). Apical media was withdrawn at day 2 and cells were differentiated at an air-liquid interface (ALI) in differentiation media for the duration of

the experiments. HC was withdrawn from the media at day 0 for a subset of cells (HC0). Control and HC0 NHBE cells formed pseudostratified epithelial tissues with ciliated and secretory surface cells (408). The mean transepithelial resistance (TER) was >700 $\Omega \cdot cm^2$ for all epithelia after 1 week of ALI culture.

Differentiated NHBE cells were mounted in Ussing chambers and pretreated with benzamil and $CFTR_{inh}$-172 to inhibit basal Na^+ absorption and anion secretion through ENaC and CFTR, respectively. Control cells stimulated by apical addition of 20 μM Uridine-5'-triphosphate (UTP) exhibited a rapid decrease in I_{SC} followed by a similarly rapid return and overshoot of the basal current (Fig 1A). Addition of the calcium-activated chloride channel inhibitor AO1 ($CaCC_{inh}$-AO1) decreased I_{SC} after stimulation with UTP. Pretreatment with paxilline, a selective blocker of high-conductance Ca^{2+}-activated K^+ channels ($K_{Ca}1.1$, BK) (416), abolished the rapid decrease in I_{SC} immediately after stimulation with UTP (Fig1B). Pretreatment with TRAM34, a selective blocker of intermediate-conductance Ca^{2+}-activated K^+ channels ($K_{Ca}3.1$, IK), had no effect on I_{SC}. In contrast, HC0 cells stimulated by apical addition of UTP exhibited only an increase in I_{SC} (Fig 1C) and pretreatment with paxilline had no effect on the current (Fig 1D). Control and HC0 cells exhibited a similar increase in $[Ca^{2+}]_i$ after stimulation with UTP as determined by Fura2-AM imaging (Fig 1F). This result indicated that purinergic receptor signaling in HC0 cells was intact and suggested that the absence of paxilline-sensitive I_{SC} is due to a more direct effect on BK channel function.

NHBE Control and HC0 cells express BK subunits

We investigated the hypothesis that reduced transcription of BK mRNAs may have been responsible for the absence of BK channel function in HC0 cells. qRT-PCR analysis of BK channel subunit mRNA expression revealed significantly reduced levels of KCNMA1 (BKα), KCNMB4 ($BK\beta_4$), and LRRC26 (a γ subunit that dramatically shifts the voltage-dependence of BK channels) in HC0 cells compared to controls (Fig 2A). NHBE cells did not exhibit detectable expression of KCNMB1 or KCNMB2 transcripts. Protein expression of BK subunits did not appear to be different between control and HC0 epithelia in both abundance and isoform, which suggested that

differential BK channel isoform expression was not the underlying cause of the HC0 phenotype (Fig 2B). No protein was detected for BKβ_4. Immunocytochemistry revealed the localization of BKα at both apical and cilia-formed membrane domains (Fig 2C). BKα exhibited a similar localization pattern as previously demonstrated for ENaCα and α-tubulin in the cilia of differentiated NHBE cells (408). Moreover, BKα was also detected in the apical membrane of surface cells and a similar pattern was observed in HC0 cells.

Differentiated NHBE cells have distinct P2YR signaling domains

UTP is an agonist of G_q-coupled $P2Y_2$ and $P2Y_4$ receptors. However, $P2Y_2$ but not $P2Y_4$ receptors are also activated by ATP (163, 257). qRT-PCR analysis revealed expression of P2RY2, P2RY4 and P2RY6 mRNA transcripts in differentiated NHBE cells (Fig 3A). However, HC0 cells did not express detectable levels of P2RY4 mRNAs. Previous experiments performed in Xenopus oocytes suggested differential regulation of BK channels by $P2Y_2$ and $P2Y_4$ receptors (136). To examine the I_{SC} response following $P2Y_2R$ activation independently of $P2Y_4R$ stimulation, cells were treated with a non-hydrolizable analog of ATP (ATP-γ-S) which is known to activate $P2Y_2R$ (175). Control and HC0 cells stimulated with UTP exhibited no further change in I_{SC} after addition of ATP-γ-S (Fig 3B). This result was consistent with complete activation of $P2Y_2$ receptors. Control cells stimulated with ATP-γ-S alone exhibited a rapid decrease in I_{SC} that was paxilline-sensitive (Fig 3C, D). Addition of UTP after stimulation by ATP-γ-S resulted in a further decrease in I_{SC}. This result suggested that there are distinct pools of $P2Y_2$ and $P2Y_4$ receptors coupled to BK that can be activated by different nucleotide triphosphates. A similar graded response to UTP after ATP-γ-S stimulation was observed in HC0 cells, although the magnitude was significantly reduced (Fig 3E, F). Western blot analysis demonstrated similar amounts of $P2Y_2$ and $P2Y_4$ receptors expressed in both groups (Fig 3G, H), despite undetectable amounts of P2RY4 transcripts in HC0 epithelia. Immunocytochemistry confirmed distinct localization patterns for $P2Y_2R$ and $P2Y_4R$ in differentiated NHBE cells. $P2Y_2$ co-localized with BKα in the cilia and on the apical membrane of surface cells (Fig 3I), whereas $P2Y_4$ receptors were only

detected at the apical membrane and not within the cilia. This result showed that there are both overlapping and unique signaling regions at the apical border of differentiated NHBE surface cells that differentially harbor specific $P2Y_2$ and $P2Y_4$ receptors.

The BK activator NS11021 potentiates the effect of UTP

Although HC0 cells appeared to express the necessary subunits of BK to produce functional channels (Fig 2), we were unable to detect BK channel activity following stimulation with UTP or ATP-γ-S. To determine if BK channels in the apical membrane of HC0 cells could be potentially activated independently of purinoceptor stimulation, we treated cells with the small molecule BK activator NS11021. Control cells stimulated with 10uM NS11021 alone exhibited a paxilline-sensitive decrease in I_{SC} (Fig 4A). In contrast, HC0 cells stimulated with NS11021 showed no change in paxilline-sensitive I_{SC} (Fig 4B). Control epithelia pretreated with NS11021, then stimulated with UTP displayed a significantly prolonged response, but no difference in magnitude compared to UTP alone (Fig 4C, E, F). This result was consistent with NS11021 altering channel gating kinetics in the presence of UTP (26). Interestingly, HC0 cells pretreated with NS11021, then stimulated with UTP, now displayed a rapid decrease in I_{SC} however the magnitude was significantly less than that observed in control epithelia (Fig 4D, E). This observation demonstrated that HC0 cells express BK in the apical membrane, though endogenous agonists like UTP cannot activate them unless previously primed by the actions of a BK channel opener.

PKC activity is required for UTP stimulated BK activity

Post-translational modification of BK alters channel activation and conductance (195). Phosphorylation at consensus sequences within the C-terminus of BKα is known to alter channel activity (365, 399). We hypothesized that if phosphorylation of BK by PKC was permissive to channel activation, inhibition of PKC activity may abolish paxilline-sensitive currents under control conditions. Pretreatment (20 mins) of control epithelia with 500 nM GF109203x, a pan-specific PKC inhibitor with greater selectivity for PKCα and PKCβ1, eliminated the paxilline-sensitive decrease in I_{SC} after stimulation

with UTP (Fig 5A, B). UTP stimulation resulted in an increase in I_{SC} that was reduced incrementally by DIDS, then $CaCC_{inh}$-AO1. Pretreatment with Gö6983, another pan-specific PKC inhibitor, had the same effect as GF109203x, providing additional pharmacological evidence of a role for PKC in BK activation. HC0 cells pretreated with either GF109203x or Gö6983 had no effect on UTP stimulated I_{SC}. Since PKC antagonism abolished BK activation, we examined whether PKC activation by phorbol ester would enhance BK function in HC0 cells. However, HC0 pretreatment with 100 nM PMA had no effect on I_{SC} (Fig 5C). Similarly, PMA did not potentiate the stimulatory effects of UTP on control cells. This result suggested that BK is already maximally phosphorylated by PKC in control epithelia. qRT-PCR analysis of specific PKC isoform transcripts revealed significantly reduced expression of PRKCA, PRKCD, and PRKCE transcripts in HC0 cells compared to control (Fig 5D). However, protein expression of PKCα, PKCδ, and PKCε appeared to be similar in control and HC0 epithelia, potentially limiting differential PKC isoform expression as a major contributor to the HC0 transport phenotype.

Hydrocortisone reduces STREX-variant KCNMA1 transcription

Alternative exon splicing and the consequent effects on channel phosphorylation is a fundamental control mechanism of BK activity. Previous studies have demonstrated PKA-mediated channel inhibition for BK channels expressing the 59-amino acid STREX exon (366). Furthermore, direct application of glucocorticoids to bovine chromaffin cells decreased STREX inclusion in KCNMA1 (197). We hypothesized HC0 epithelia were expressing the STREX splice-variant of KCNMA1 that resulted in greatly reduced PKC-mediated BK channel activation. We designed qRT-PCR primer-probe sets that target the STREX inclusion site in full-length KCNMA1 (nucleotides≈2300-2600). Detection with these primer-probe sets would indicate inclusion of the STREX exon, while no detection would indicate KCNMA1 transcripts lacking the STREX region. qRT-PCR analysis of differentiated epithelia revealed statistically greater expression of KCNMA1 transcripts containing the STREX exon in HC0 cells compared to controls (Fig 5F). This result suggested that HC0 epithelia express a greater number of BK channels containing

the PKA-inhibited STREX exon and therefore are not activated by physiologic stimulation (Fig 7).

Differentiated NHBE express CaCCs as well as ClC chloride channels

NHBE cells stimulated with UTP exhibit a paxilline-sensitive K^+ current as well as $CaCC_{inh}$-AO1-sensitive anion secretion. We further characterized anion secretion as having a DIDS-sensitive component in addition to a $CaCC_{inh}$-AO1-sensitive I_{SC} (Fig 5A). DIDS is known to inhibit ClC-Ka/b chloride channels and qRT-PCR analysis revealed that control and HC0 cells express mRNAs for CLC-Ka, CLC-Kb and the necessary channel forming β-subunit barttin (Fig 6A). Differentiated cells also expressed mRNAs that encode the Ca^{2+}-activated chloride channel ANO1 (TMEM16a). Western blot analysis demonstrated protein expression of TMEM16a, ClC-Kb, and Barttin but not ClC-Ka (Fig 6B). Interestingly, immunocytochemistry revealed TMEM16a localization in the apical membrane of surface cells but not within cilia (Fig 6C). This result is consistent with the observation of distinct signaling and transport domains within the apical membrane and cilia of differentiated surface cells.

Discussion

Large-conductance, Ca^{2+}-activated K^+ channels ($K_{Ca}1.1$, BK) are ubiquitously expressed and support numerous and diverse physiologic functions. Recent studies revealed BK channel expression within the apical membrane of freshly isolated bronchial epithelial cells, where they participate in mucociliary clearance by sustaining the driving force for anion efflux across the apical membrane (223). In the present study, I_{SC} measurements confirmed the presence of an ATP/UTP stimulated-current that was inhibited by the presence of a selective large-conductance Ca^{2+}-activated K^+ channel blocker, paxilline. The presence of a UTP stimulated, paxilline-sensitive current was demonstrated in differentiated pseudostratified epithelia derived from NHBE cells. Stimulation with UTP was previously shown to activate Ca^{2+}-activated Cl^- channels in airway epithelial cells (294) and a similar conclusion is supported in the present study based on activation of a $CaCC_{inh}$AO1-sensitive anion current by UTP. Interestingly,

NHBE cells differentiated in the absence of HC (HC0) do not exhibit a paxilline-sensitive current after UTP stimulation, displaying only an increase in $CaCC_{inh}AO1$-sensitive I_{SC}. Moreover, Fura2-AM measurements of $[Ca^{2+}]_i$ confirmed similar increases in cytoplasmic $[Ca^{2+}]$ after UTP stimulation in control and HC0 cells. This result suggested that P2YR/G_q-coupled Ca^{2+} mobilization remains intact in HC0 cells even though a Ca^{2+}-activated BK current was not detected.

Functional BK channels consist of large, membrane-spanning α-subunits that combine with auxiliary β- and γ-subunits that are expressed in a tissue specific manner (183, 328). These auxiliary subunits modulate the biophysical properties of BK channels, allowing their use in diverse physiologic systems. We initially hypothesized that differential expression of β- and γ-subunits in control and HC0 epithelia could account for the observed variation in UTP stimulated BK activation. HC0 cells expressed significantly fewer transcripts of BKα, β_4 and the γ-subunit LRRC26 compared to control as determined by qRT-PCR. However, western blot analysis revealed no difference in protein expression, which suggested varied BK subunit-composition does not completely explain altered BK channel activity in HC0 cells.

Although UTP evoked a similar $[Ca^{2+}]_i$ increase in both control and HC0 conditions, our results did not eliminate the possibility of BK inhibition by Ca^{2+}-independent purinergic receptor stimulated signaling pathways. Numerous studies have demonstrated inhibitory effects of purinergic receptors on ion channels. In distal nephron epithelia, stimulation of $P2Y_2$-receptors reduced ENaC activity by enhancing PIP_2 metabolism (277, 278). Moreover, ATP stimulation inhibited KCNQ1 in dark cells of the inner ear, ROMK2 channels in the distal nephron, and BK activity in spermatogenetic cells (214, 227, 236, 395). Additionally, co-expression experiments performed in *Xenopus laevis* oocytes revealed differential BK regulation by $P2Y_2R$ and $P2Y_4R$(136). Oocytes exposed to UTP expressing only $P2Y_4R$ led to increased BK current evoked by membrane depolarization, while oocytes expressing only $P2Y_2R$ displayed reduced BK current. Based on these findings, we investigated the hypothesis that differential expression of P2Y-receptors by control and HCO cells may be responsible for the differences in BK channel activation produced by UTP. Control and HC0 cells were

shown to express similar amounts of $P2Y_2$ and $P2Y_6$ transcripts as determined by qRT-PCR. However, $P2Y_4R$ mRNA was only detected in control cells but not HC0 epithelia. Ussing chamber experiments suggested the existence of two distinct pools of P2Y-receptors coupled to BK activation in control epithelia. UTP is an agonist for both $P2Y_2$ and $P2Y_4$ receptors, while ATP activates $P2Y_2$Rs but not $P2Y_4$Rs. Cells stimulated with ATP-γ-S, a non-hydrolyzable analog of ATP, displayed a paxilline-sensitive decrease in I_{SC} but could be further stimulated with UTP, suggesting the presence of non-activated UTP-sensitive, but not ATP-sensitive, P2Y-receptors. However, ATP-γ-S could not evoke additional currents in epithelia previously stimulated by UTP. This result indicated the presence of UTP-sensitive, but not ATP-sensitive, P2Y-receptors in differentiated NHBE cells and suggested that a maximal dose of UTP was able to activate apical $P2Y_2$ and $P2Y_4$ receptors, while a maximal dose of ATP-γ-S was only able to stimulate $P2Y_2$ receptors. Nonetheless, UTP and ATP-γ-S had similar effects on BK activation, suggesting that selective activation of $P2Y_2R$ and $P2Y_4R$ does not differentially regulate BK in airway epithelia. Furthermore, UTP and ATP-γ-S had functionally similar effects on I_{SC} in HC0 cells, which indicated that differential purinergic receptor activation could not account for the absence of BK currents observed in HC0 cells.

Curiously, UTP stimulation after exposure to ATP-γ-S resulted in an increase in I_{SC} in HC0 cells, suggesting the presence of apical $P2Y_4$ receptors despite undetectable amounts of mRNA. Western blot analysis demonstrated similar levels of $P2Y_2$ and $P2Y_4$ protein expression in control and HC0 cells, confirming $P2Y_4$ expression in HC0 epithelia. It is worth noting that NHBE cells were exposed equally to HC during *in vitro* expansion and growth before differentiation. Therefore, it is likely that $P2Y_4R$ protein expressed during the first week of differentiation is subject to limited turnover in the apical membrane of differentiated NHBE cells and consequently can be detected by western blot in HC0 cells despite apparent regulation of P2RY4 transcription by HC (34).

Our results indicated comparable BK channel expression as well as intact and functionally similar $P2Y_2$ and $P2Y_4$ mediated signaling in both control and HC0 cells, despite markedly different I_{SC} responses to purinergic stimulation between the two

groups. Since we were unable to activate BK through physiologically relevant signaling pathways in HCO epithelia but were able to detect BK protein by western blot, we explored the possibility that BK was not expressed on the surface of HC0 cells. The small molecule BK opener NS11021 allows for channel activation at more negative potentials by altering BK gating kinetics without affecting single-channel conductance (26). Furthermore, NS11021 has previously been shown to evoke a ΔI_{SC} response in airway epithelial cells (224). Therefore, we used NS11021 to test the hypothesis that HC0 cells did not express functional BK channels in the apical membrane. Control cells exposed to NS11021 demonstrated a decrease in I_{SC} consistent with previous reports. NS11021 had no effect on I_{SC} in HC0 epithelia. However, HC0 cells treated with NS11021 before stimulation with UTP displayed a paxilline-sensitive decrease in I_{SC}, demonstrating that it is possible to activate BK channels in the apical membrane of HC0 epithelia.

BK activation occurs by membrane depolarization and increased cytoplasmic [Ca^{2+}]. Biophysical experiments have revealed complex allosteric interactions between voltage-sensing and Ca^{2+}-sensing domains (VSD and RCK, respectively) in BKα, where activation of the two RCK domains differentially alter the VSD and pore opening (312). Since we observed UTP stimulated BK activity by HC0 cells only in the presence of NS11021, it is reasonable to speculate that NS11021 may have had a similar allosteric effect on BK, making the channel more sensitive to activation by $[Ca^{2+}]_i$ or membrane depolarization. Allosteric effects could also explain the potentiation of UTP stimulated BK activity displayed by control epithelia exposed to NS11021. As previously mentioned, NS11021 alters BK gating kinetics and to this point we observed significantly longer, but not larger, BK currents in control cells exposed to the opener molecule. Furthermore, NS11021 alone evoked paxilline-sensitive currents in control but not HC0 epithelia, suggesting that BK expressed in HCO cells have an altered basal sensitivity to channel activation by NS11021.

Posttranslational modification of BKα by phosphorylation is another well studied regulator of BK activity. In the present study, control cells pretreated with the pan-specific PKC inhibitor GF109203x no longer displayed a UTP stimulated paxilline-

sensitive current, but did show an increase in I_{SC} following UTP exposure. A similar effect was observed with Gö6983, another pan-specific PKC inhibitor. These results established that PKC-dependent phosphorylation was necessary and permissive for BK activation following purinergic stimulation. Further evidence to this point was the absence of BK activation by UTP in HC0 cells pretreated with PMA, an activator of PKC that has previously been shown to stimulate BK current (418). Our finding that PMA was unable to potentiate paxilline-sensitive currents in control epithelia or activate BK in HC0 epithelia suggests that BK is already maximally phosphorylated by PKC. Control and HC0 epithelia were shown to express statistically different amounts of PKCα, δ, and ε, isoforms but did not express different quantities of protein. Therefore, differential expression of PKC isoforms does not account for the lack of BK channel activation observed in HC0 epithelia.

Tissue specific KCNMA1 splicing is another regulatory mechanism that can dramatically alter BK channel function. α-subunit splice-variants containing the "STREX" (stress axis-regulated) exon have been shown to exhibit enhanced Ca^{2+} sensitivity and PKA-mediated regulation (366). In bovine chromaffin cells, for example, glucocorticoid exposure decreased STREX inclusion in BK channels (197). We hypothesized that HC0, but not control, epithelia express the STREX variant BKα. qRT-PCR confirmed increased expression of STREX containing KCNMA1 transcripts in HC0 cells. Furthermore, mRNAs for native-KCNMA1 and STREX-KCNMA1 were inversely correlated, indicating that total KCNMA1 transcription is similar in both conditions but the stoichiometry of the specific splice-variants are distinct. Functional BK channels contain four α-subunits that demonstrate cooperativity during activation (312, 348). Insertion of non-stimulatory PKA-inhibited α-subunits (STREX-KCNMA1) may reduce cooperativity and the conformational-changes required for channel activation by purinergic signaling. A previous study demonstrated BK channel inhibition by a single STREX subunit and concluded that all α-subunits must be phosphorylated by PKA for activation (365). Therefore, it appears likely that increased expression of STREX-containing α-subunits in the absence of HC inhibits BK activation in HC0 epithelia.

Additionally, differentiated control and HC0 cells expressed ClC-Kb chloride channels in addition to ANO1 and CFTR. Both control and HC0 epithelia possessed a DIDS-sensitive current and a $CaCC_{inh}AO1$-sensitive current, suggesting the presence of multiple anion channels in the apical membrane. ClC-K channels require an auxiliary β-subunit, barttin, for membrane expression and loss of channel function in renal epithelia is associated with type 3 Bartter syndrome (7, 63). ClC-K + barttin channel expression has previously been reported in Calu-3 cells, a human airway submucosal gland cell model (245). Our results indicate that differentiated primary airway epithelial cells also express specific ClC chloride channels.

Finally, we have previously demonstrated cilia-localization of important ion channels and receptors in differentiated NHBE cells (408). In the present study, BKα and $P2Y_2$ receptors were shown to co-localize within the cilia while BKα, $P2Y_2$, and $P2Y_4$ receptors co-localize at the apical membrane. This result suggests there are unique signaling regions at the apical border of airway surface cells that differentially harbor specific receptors and ion channels. AN01, for example, exists only at the apical surface of the cell but not within cilia. During the progression of many airway diseases, the distribution and quantity of surface ciliated and secretory cells becomes unbalanced (182, 336). Loss of ciliated cells and an increase of mucin secreting cells is termed mucous or goblet cell metaplasia and is a common manifestation in asthma and COPD(336). Therefore, it is possible that the loss of functionally distinct signaling domains caused by airway remodeling may also contribute to the reduced MCC observed in large airway diseases.

Summary and conclusions

Previously, we investigated the role of glucocorticoid receptor stimulation on the differentiation of NHBE cells and the development of ion transport pathways essential to MCC (408). In the present study, we extended these investigations by examining the effects of hydrocortisone on purinergic receptor-stimulated ion transport, an important autocrine signaling pathway that regulates ASL depth. The key findings of this study include 1) HC is required for BK activation by purinergic receptor agonists, 2) HC

reduces STREX exon insertion in KCNMA1, permitting PKC -mediated channel activation, 3) overlapping and unique purinergic signaling regions exist at the apical border of differentiated surface cells, and 4) BK channels localize in the cilia of surface cells. An overview of the differential response to purinoceptor-stimulated BK activation in control and HC0 epithelia is summarized in Figure 7. Control and HC0 epithelia display a similar increase of $[Ca^{2+}]_i$ after exposure to UTP, which causes opening of Ca^{2+}-activated chloride channels (ANO1/TMEM16A). However, HC0 epithelia display no BK current after UTP stimulation, likely due to increased expression of STREX-variant KCNMA1, which is upregulated in the absence of HC. Previous work has demonstrated an inhibitory effect of STREX on BK channel activation (93, 272, 366). A key mechanism responsible for channel inhibition has been linked to PKA-dependent phosphorylation of specific cites residing within the STREX sequence (365). In the present study, basal CFTR function suggests tonic PKA activity which could lead to inhibition of STREX-variant BK channels expressed in HC0 epithelia. Additionally, STREX-variant BK channels have been shown to have altered responsiveness to PKC regulation (414). Here, we demonstrated permissive and necessary regulation of BK activation by PKC. Therefore, altered regulation by PKC-mediated phosphorylation may have also contributed to the loss of BK activation in HC0 cells. The results presented here establish a previously unrecognized role of glucocorticoids in purinergic regulation of BK channels in airway epithelial cells.

Figure 3.1

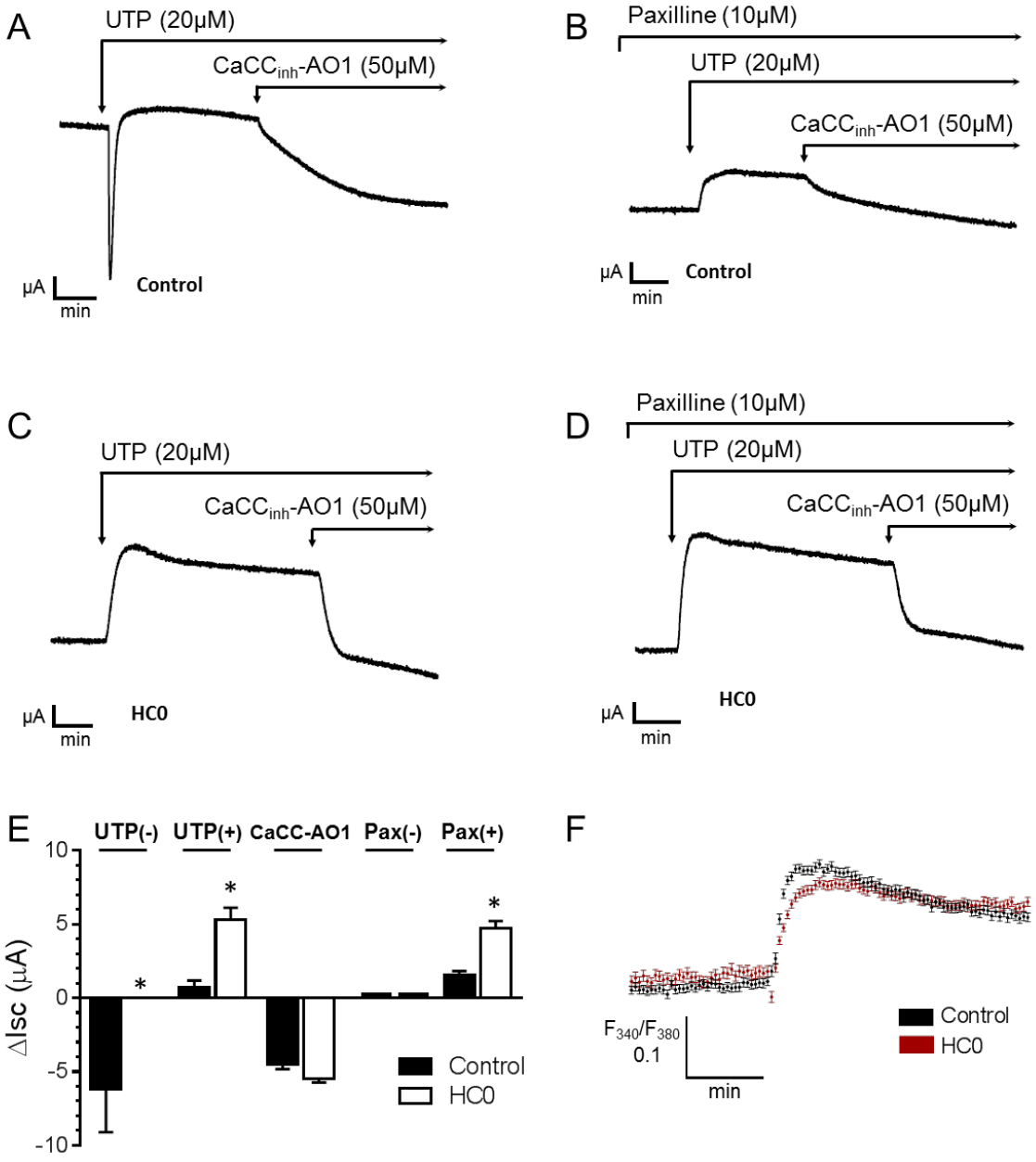

A
UTP (20μM)
CaCC$_{inh}$-AO1 (50μM)
μA
min
Control
B
Paxilline (10μM)
UTP (20μM)
CaCC$_{inh}$-AO1 (50μM)
μA
min
Control
C
UTP (20μM)
CaCC$_{inh}$-AO1 (50μM)
μA
min
HC0
D
Paxilline (10μM)
UTP (20μM)
CaCC$_{inh}$-AO1 (50μM)
μA
min
HC0
E
UTP(-)
UTP(+)
CaCC-AO1
Pax(-)
Pax(+)
10
5
0
-5
-10
ΔIsc (μA)
*
Control
HC0
F
F$_{340}$/F$_{380}$
0.1
min
Control
HC0

Figure 3.1. NHBE cells exhibit paxilline-sensitive K^+ secretion after stimulation with UTP that was not observed in HC0 cells. (a, d) Representative I_{SC} traces of control (a, b) and HC0 (c, d) epithelia pretreated with 5uM benzamil and 20uM $CFTR_{inh}$-172 then stimulated with UTP (n=6). Addition of 50 μM $CaCC_{inh}$-AO1 decreased I_{SC} after stimulation with UTP. Control monolayers pretreated with 10 μM paxilline display only anion secretion after stimulation with UTP (b). Paxilline had no effect on the UTP-stimulated I_{SC} in HC0 epithelia (d). (e) Histogram summarizing I_{SC} results represented in (a, d). UTP(-) refers to the decrease in I_{SC} after stimulation with UTP. Pax (-) refers to a decrease in I_{SC} after stimulation with UTP for epithelia pretreated with paxilline. Statistical significance (*, $p<0.05$) represents difference between similar control and HC0 I_{SC} measurements determined by t-tests. (f) Fura2-AM F_{340}/F_{380} ratio of control and HC0 epithelia stimulated with UTP (n=25 cells).

Figure 3.2

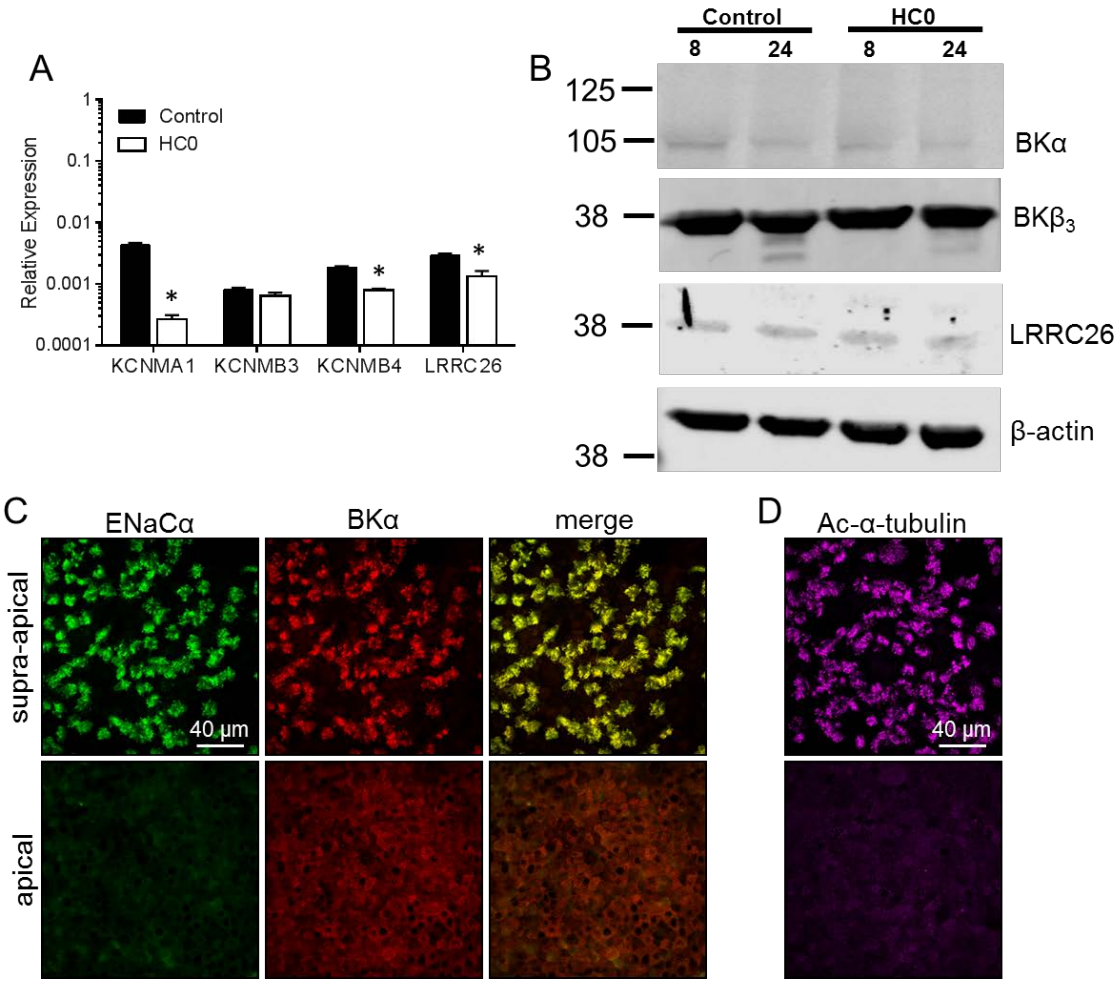

A
Relative Expression
1
0.1
0.01
0.001
0.0001
Control
HC0
*
*
*
KCNMA1
KCNMB3
KCNMB4
LRRC26
B
Control
HC0
8
24
8
24
125
105
38
38
38
BKα
BKβ3
LRRC26
β-actin
C
ENaCα
BKα
merge
supra-apical
apical
40 μm
D
Ac-α-tubulin
40 μm

Figure 3.2. Differentiated NHBE cells express the α_1, β_3, β_4 and LRRC26 γ subunits of BK (K_{Ca}1.1) potassium channels. (a) qRT-PCR analysis of BK channel subunit mRNAs in control and HC0 epithelia after 8 days of differentiation (n=6). Expression is relative to GapDH (1.0). (b) Western blot analysis of BKα, β_4 and LRRC26 γ-subunit in day 8 and day 24 control and HC0 epithelia. (c) Immunocytochemistry showing co-localization of ENaCα and BKα in the cilia of differentiated control NHBE cells. (d) Immunocytochemistry demonstrating localization of acetylated α-tubulin, staining motile cilia on the surface of differentiated control NHBE cells. Statistical significance (*, $p<0.05$) represents difference between relative expression of mRNAs in control and HC0 cells determined by t-tests.

Figure 3.3

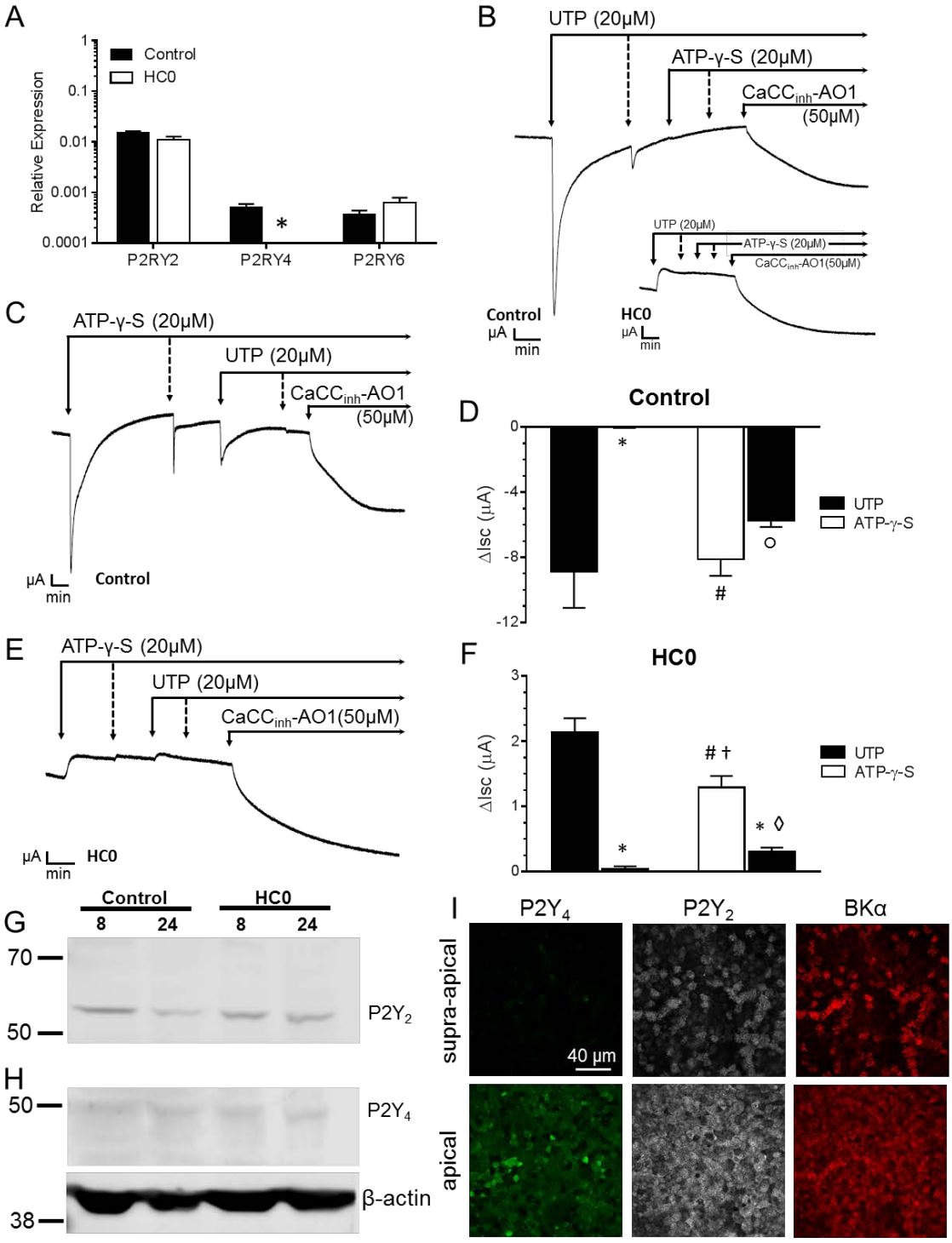

A
Control
HC0
Relative Expression
1
0.1
0.01
0.001
0.0001
*
P2RY2
P2RY4
P2RY6
B
UTP (20μM)
ATP-γ-S (20μM)
CaCC$_{inh}$-AO1
(50μM)
UTP (20μM)
ATP-γ-S (20μM)
CaCC$_{inh}$-AO1(50μM)
Control
μA
min
HC0
μA
min
C
ATP-γ-S (20μM)
UTP (20μM)
CaCC$_{inh}$-AO1
(50μM)
μA
min
Control
D
Control
ΔIsc (μA)
0
-4
-8
-12
*
#
o
UTP
ATP-γ-S
E
ATP-γ-S (20μM)
UTP (20μM)
CaCC$_{inh}$-AO1(50μM)
μA
min
HC0
F
HC0
ΔIsc (μA)
3
2
1
0
†
* ◊
*
UTP
ATP-γ-S
G
Control
HC0
8
24
8
24
70
50
P2Y$_2$
H
50
P2Y$_4$
β-actin
38
I
P2Y$_4$
P2Y$_2$
BKα
supra-apical
apical
40 μm

Figure 3.3. NHBE cells express $P2Y_4$ receptors in the apical membrane, forming a pool of P2YR that are not stimulated by ATP-γ-S. (a) qRT-PCR analysis of P2YR mRNAs in control and HC0 monolayers after 8 days of differentiation (n=6). Statistical significance (*, $p<0.05$) represents differences between relative expression of mRNAs in control and HC0 cells determined by t-tests. (b) Representative I_{SC} traces of control and HC0 (inset) epithelia stimulated with 20 μM UTP then 20 μM ATP-γ-S (n=6). (c, d) Representative I_{SC} traces of control (c) and HC0 (d) epithelia stimulated with 20 μM ATP-γ-S then 20 μM UTP (n=6). (e, f) Histogram summarizing effects of UTP and ATP-γ-S on I_{SC} in control (e) and HC0 (f) epithelia. Initial stimulant is represented as left bar in each pair. Statistical significance ($p<0.05$) between initial and post stimuli marked by (*), between primary stimuli marked by (†), between post stimuli marked by (○), between UTP bars marked by (◊), between ATP-γ-S bars marked by (#). (g, h) Western blot analysis of $P2Y_2R$ (g) and $P2Y_4R$ (h) in day 8 and day 24 control and HC0 epithelia. (i) Immunocytochemistry showing the co-localization of $P2Y_2$ and BKα in the cilia of control differentiated NHBE cells. $P2Y_4$ is only expressed in the apical membrane and not within the cilia.

Figure 3.4

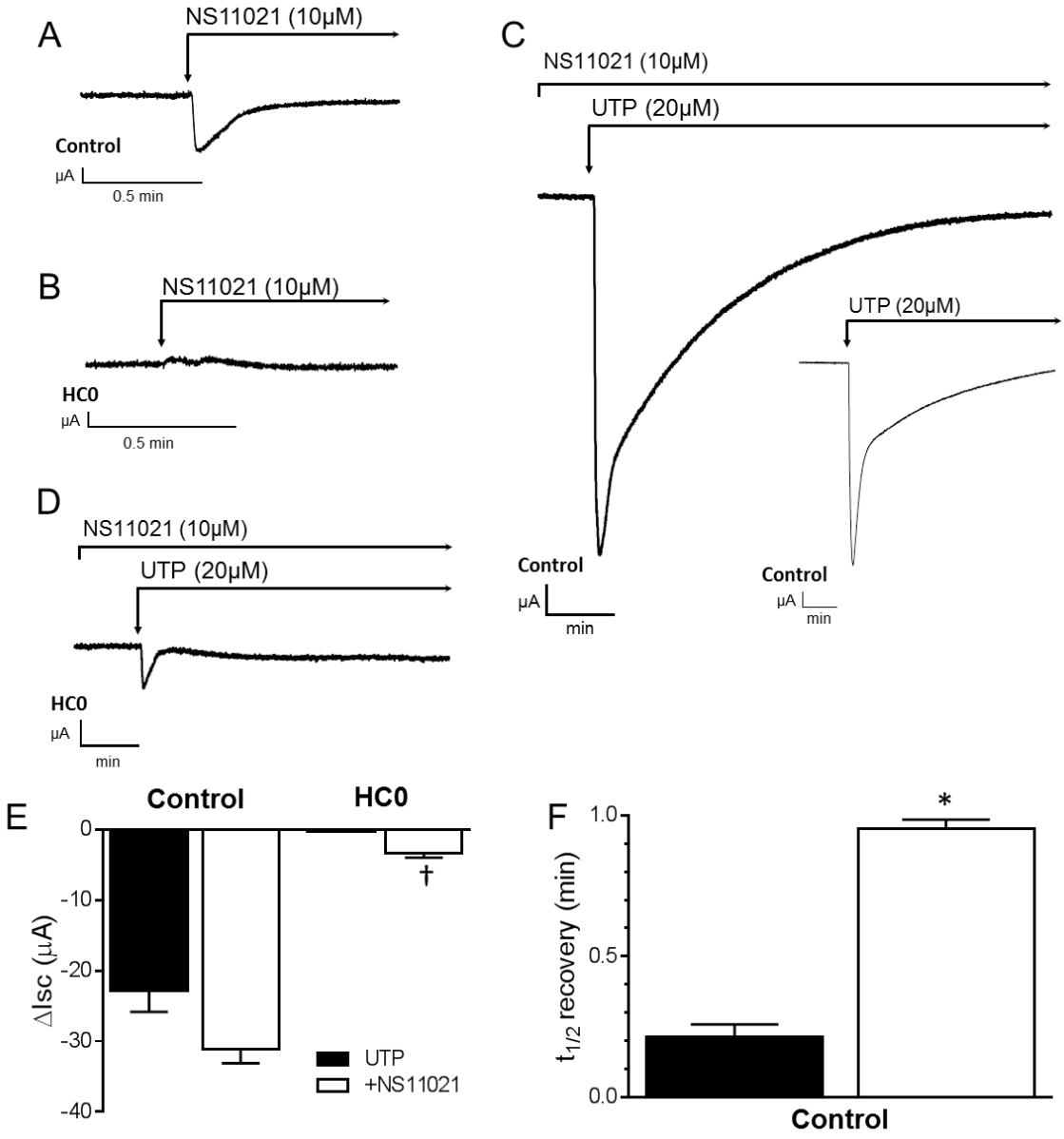

A
NS11021 (10μM)
Control
μA
0.5 min
B
NS11021 (10μM)
HC0
μA
0.5 min
C
NS11021 (10μM)
UTP (20μM)
UTP (20μM)
Control
μA
min
Control
μA
min
D
NS11021 (10μM)
UTP (20μM)
HC0
μA
min
E
Control
HC0
0
-10
-20
-30
-40
ΔIsc (μA)
†
UTP
+NS11021
F
1.0
0.5
0.0
$t_{1/2}$ recovery (min)
*
Control

Figure 3.4. The small molecule BK activator NS11021 potentiates the stimulatory effect of UTP by increasing the duration of the I_{SC} response. (a, b) Representative I_{SC} traces of control (a) and HC0 (b) epithelia stimulated with 10 μM NS11021 (n=6). (c, d) Representative I_{SC} traces of control (c) and HC0 (d) monolayers pretreated with 10 μM NS11021 then stimulated with 20 μM UTP (n=6). Representative I_{SC} trace of control epithelia stimulated with 20 μM UTP (c, inset). (e) Histogram summarizing the results represented in (c, d). Statistical significance (†, $p<0.05$) represents the difference between UTP + NS11021 in control and HC0 epithelia determined by one-way ANOVA. (f) Histogram summarizing the time to I_{SC} recovery to half of baseline following stimulation with UTP in control epithelia (c). Statistical significance (*, $p<0.05$) represents difference in $t_{1/2}$ between UTP and UTP + NS11021 control monolayers as determined by t-tests.

Figure 3.5

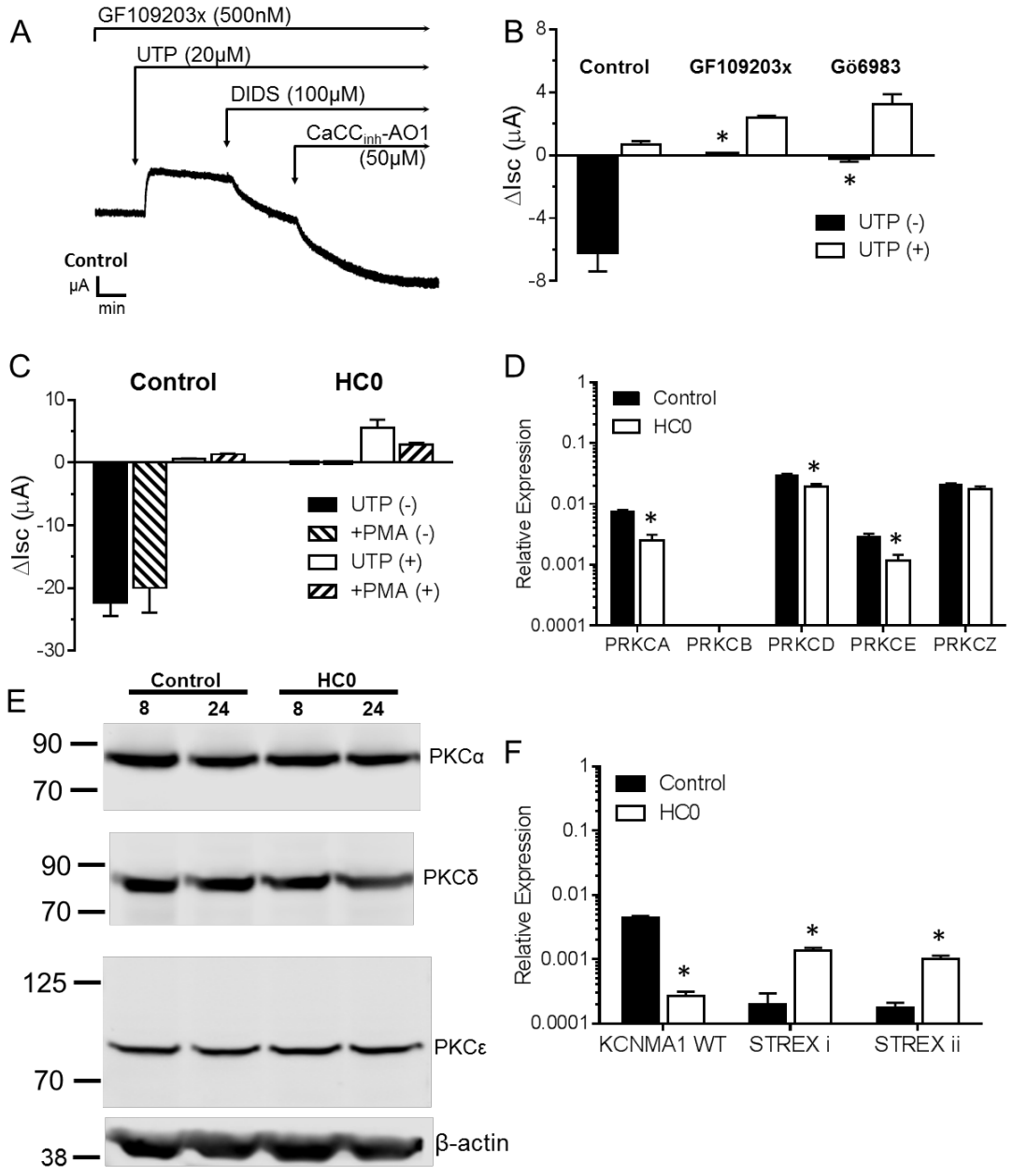

A
GF109203x (500nM)
UTP (20μM)
DIDS (100μM)
CaCC$_{inh}$-AO1 (50μM)
Control
μA
min
B
Control
GF109203x
Gö6983
ΔIsc (μA)
UTP (-)
UTP (+)
C
Control
HC0
ΔIsc (μA)
UTP (-)
+PMA (-)
UTP (+)
+PMA (+)
D
Control
HC0
Relative Expression
PRKCA
PRKCB
PRKCD
PRKCE
PRKCZ
E
Control
HC0
8
24
8
24
PKCα
PKCδ
PKCε
β-actin
F
Control
HC0
Relative Expression
KCNMA1 WT
STREX i
STREX ii

Figure 3.5. PKC inhibition blocks BK activation by UTP. (a) Representative I_{SC} trace of control epithelia pretreated (20 min) with 500 nM GF109203x, a pan-specific PKC inhibitor (particularly effective against PKCα and β1), then stimulated with 20 μM UTP (n=6). Addition of 100 μM DIDS then 50 μM $CaCC_{inh}$-AO1, each reduced the I_{SC}. (b) Histogram summarizing the effects of PKC inhibitors GF109203x and Gö6983 on UTP stimulated I_{SC}. (c) Histogram summarizing the effects of 100 nM PMA on UTP stimulated I_{SC} in control and HC0 epithelia. (d) qRT-PCR analysis of PKC isoform mRNAs (n=6). Statistical significance (*, $p<0.05$) represents differences between relative expression of mRNAs in control and HC0 cells determined by t-tests. (e) Western blot analysis of PKCα, δ and ε in day 8 and day 24 control and HC0 epithelia. (f) qRT-PCR analysis of KCNMA1 splice variants (n=6). KCNMA1 WT is previously shown in Figure 2a. STREXi and STREXii are KCNMA1 splice variants containing the STREX exon.

Figure 3.6

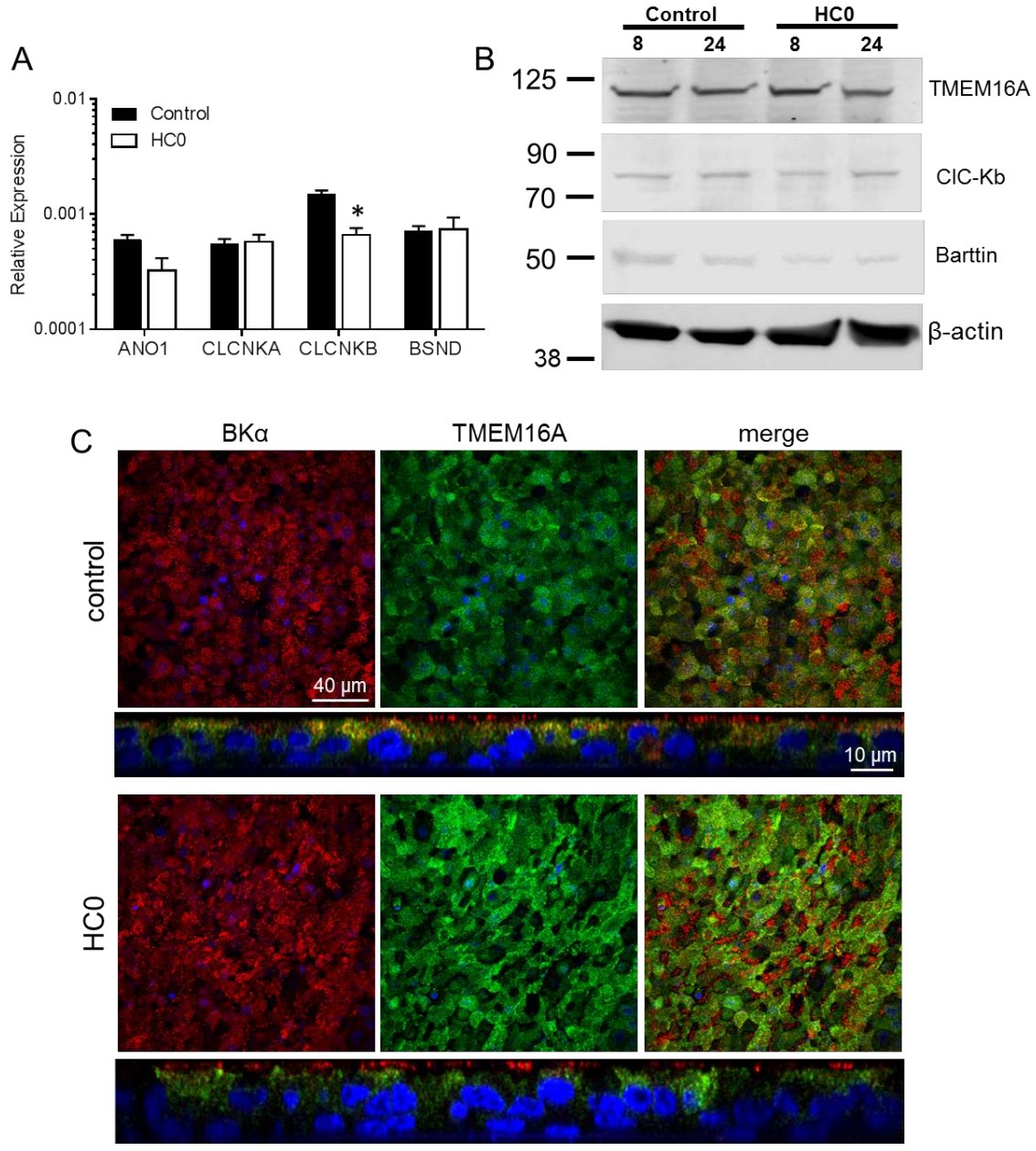

A
Relative Expression
0.01
0.001
0.0001
Control
HC0
*
ANO1
CLCNKA
CLCNKB
BSND
B
Control
HC0
8
24
8
24
125
90
70
50
38
TMEM16A
ClC-Kb
Barttin
β-actin
C
BKα
TMEM16A
merge
control
40 μm
10 μm
HC0

Figure 3.6. Differentiated NHBE cells express DIDS-sensitive ClC-Ka/b chloride channels as well as the calcium-activated chloride channel ANO1 (TMEM16A). (**a**) qRT-PCR detection of specific chloride channel mRNAs. BSND (barttin) encodes the β-subunit required for ClC chloride channel function (n=6). Statistical significance (*, $p<0.05$) represents difference between relative expression of mRNAs in control and HC0 cells determined by t-test. (**b**) Western blot analysis of TMEM16a, ClC-Kb, and Barttin in day 8 and day 24 control and HC0 epithelia. (**c**) Immunocytochemistry demonstrating the differential localization of BKα (in cilia and apical membrane) and ANO1 (TMEM16A) (apical only).

Figure 3.7

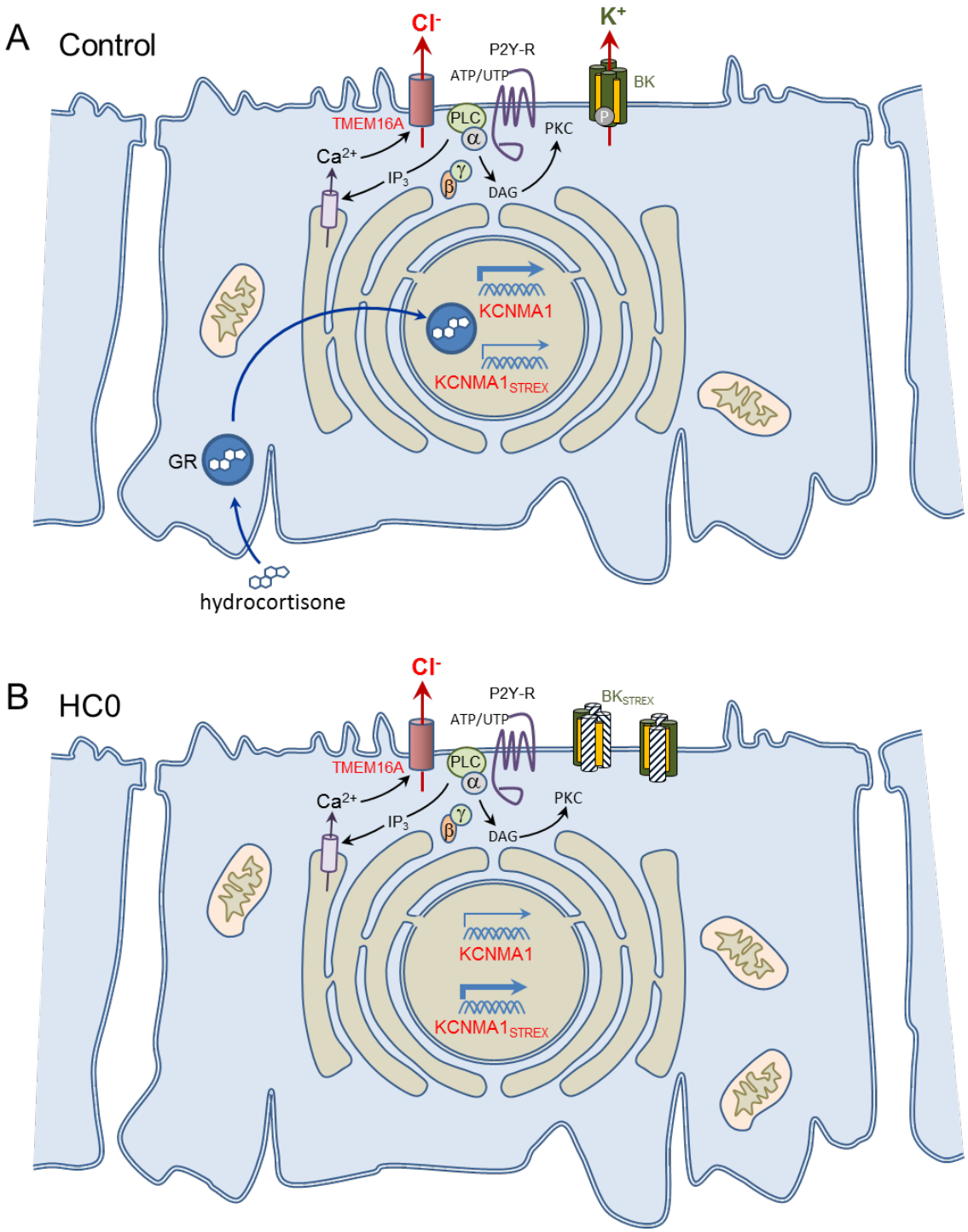

A
Control
Cl-
K+
P2Y-R
ATP/UTP
BK
TMEM16A
PLC
α
PKC
Ca2+
IP3
γ
β
DAG
KCNMA1
KCNMA1STREX
GR
hydrocortisone
B
HC0
Cl-
P2Y-R
ATP/UTP
BKSTREX
TMEM16A
PLC
α
PKC
Ca2+
IP3
γ
β
DAG
KCNMA1
KCNMA1STREX

Figure 3.7. Cell model showing the effects of ATP/UTP on specific ion transport processes in control (a) and HC0 (b) epithelia. ATP/UTP stimulate G_q-coupled purinergic receptors expressed on the apical membrane of surface airway epithelial cells leading to increased $[Ca^{2+}]_i$ and activation of PKC. PKC-dependent phosphorylation of BK permits channel activation after stimulation with ATP/UTP, which also activates Ca^{2+}-activated chloride channels (ANO1/TMEM16A). In control epithelia, HC suppresses transcription of KCNMA1 that contain the STREX exon. In HC0 epithelia, $KCNMA1_{STREX}$ mRNAs are the predominant transcript encoding BKα. The STREX exon makes BKα unresponsive to stimulatory purinergic signaling as demonstrated by the present study.

Chapter 4

Regulation of Claudin Expression and Paracellular Ion Transport by Glucocorticoids in Differentiated Human Bronchial Epithelial Cells

Nathan A. Zaidman[†], Angela Panoskaltsis-Mortari[†*] and Scott M. O'Grady[†§]

Departments of Integrative Biology and Physiology[†], Pediatrics[*], and Animal Science[§], University of Minnesota, 6-125 Jackson Hall, Minneapolis MN.

Synopsis

The structural and functional integrity of the airway epithelium is essential to maintaining health. The fence function performed by epithelial tissues is reliant on the content and stability of tight junctional complexes that form between every epithelial cell. Recent discoveries suggest that epithelial tight junctions are far more complex than previously believed, with evidence that the transmembrane protein family of Claudins form paracellular ion channels that are both size and charge selective. The contribution of these proteins to paracellular ion transport is fundamental to both absorptive and secretory epithelia, as demonstrated by the disease causing phenotypes resulting from mutation in claudin genes. In the present study, normal human bronchial epithelial cells were differentiated *in vitro* to better understand the processes leading to the development of mature tight junctions. Claudin mRNA expression was tracked throughout mucociliary differentiation and some isoforms displayed time-dependent upregulation that corresponded with the development of transepithelial resistance. Furthermore, hydrocortisone (HC) reduced paracellular permeability of Na^+ and Cl^-, which suggests HC helps to maintain the integrity and selectivity of epithelial TJs. Claudins, as well as the scaffolding protein ZO-1, localized at cellular locations besides TJs including surface cell cilia. The results of this study advance our knowledge of claudin expression in the pulmonary epithelium and expand our understanding of the cellular role of claudins and other TJ proteins.

Introduction

Intercellular junctions between adjacent epithelial cells form important connections that maintain a physical barrier separating external from internal environment (39). The paracellular space is characterized by three predominant structures: tight junctions, adherens junctions and desmosomes (279). Epithelial tight junctions are the most apically located of the three adhesion structures. They are multiprotein complexes composed of transmembrane proteins including occludin, junctional adhesion molecules (JAMs), and claudins, which are anchored internally to actin through scaffolding proteins such as ZO-1 (39). Tight junctions (TJs) form rings about the apical circumference of the epithelial cell and are the critical structure in regulating paracellular ion, water and molecular transport in epithelial tissues (133, 318). Therefore, they are critically important in supporting transepithelial transport processes essential for airway mucociliary clearance.

Claudin proteins have emerged as the key determinant of TJ charge and size selectivity since they were first discovered as a strand-forming component of TJs (108, 265). Currently, there are 27 members of the claudin gene family that encode 22-27 kDa proteins containing four transmembrane domains and two extracellular loops that interact with adjacent claudins through both homotypic and heterotypic binding (123, 187). Epithelial cells express claudins in a tissue-specific manner, allowing for remarkable heterogeneity in TJ composition and paracellular flux characteristics (265). Categorically, claudins are characterized as either pore-forming (increase permeability) or sealing-type (reduce permeability) TJ proteins. Pore-forming claudins, such as claudin-2, claudin-7 and claudin-10, are typically expressed by “leaky” (low resistance) epithelial tissues, while barrier forming claudins, such as claudin-1, claudin-5 and claudin-11, are generally considered sealing-type claudins found in epithelia with higher paracellular resistances, though this function is not universal (265). Claudins are implicated in the pathology of several human diseases. For example, familial hypomagneaemia with hypercalciuria and nephrocalcinosis (FHHNC), an autosomal recessive disorder that results in divalent cation wasting and chronic renal failure, has been linked to mutations in the claudin-16 gene (132). Additionally, mutations to claudin-14 result in nonsyndromic deafness due to

cation leak between the perilymph and endolymph in the cochlea of the ear (24). Such disorders caused by claudin dysfunction highlight the importance of well-maintained and regulated TJs for normal epithelial function.

Besides acting as an integral component of epithelial TJs, claudins have recently been shown to have additional cellular functions. Claudin-7 has been shown to recruit EpCAM into glycolipid-enriched membrane microdomains (GEMs) away from epithelial junctions and is associated with colon, pancreatic and anaplastic thyroid cancer (138, 191, 262). Claudin-1 has been implicated in susceptibility to numerous viral infections including dengue, rotavirus, HIV and respiratory syncytial virus (53, 84, 112, 254, 255, 359, 403). In hepatocytes, claudin-1 also forms a co-receptor complex with CD81 in tetraspanin enriched microdomians (TEMs), which play a critical role in hepatitis C virus (HCV) entry (95, 275). Moreover, anti-claudin 1 antibodies inhibit HCV entry into heaptocarcinoma cells (190).

Identification of specific characteristics that each claudin confers to overall permeability of the junction is difficult to determine since this depends heavily on interactions with other components of the TJ microenvironment. Much of our current knowledge of individual claudins is based on overexpression, knockout or mutagenesis studies in stable cell lines that may not be capable of recapitulating TJs in native tissues (123, 369). Studies on pulmonary epithelium have found that claudins-3, -4, -7 and -18 are the most abundant in the alveoli, while claudins-1, -3, -4, -7, and -18 are highly expressed in bronchiolar epithelial cells (69, 266). Acute regulation of TJ permeability by histamine was also demonstrated in differentiated airway cells, where addition of histamine stimulated a rapid but transient increase in paracellular Na^+ flux (102). Our recently published results demonstrated the influence of glucocorticoids (hydrocortisone (HC)) on the establishment and maintenance of ion transport properties of human bronchial epithelium during mucociliary differentiation (408). We hypothesized that glucocorticoids might also affect paracellular ion transport through regulation of claudin protein expression. In the present study we measured the expression of 20 claudin isoforms in HC exposed (control) and HC withdrawn (HC0) normal human bronchial epithelial (NHBE) cells during mucociliary differentiation. Furthermore, we

characterized the role of HC in determining the ion selectivity of the paracellular pathway. Finally, we observed claudin localization at sites outside of TJs, suggesting potential roles for claudins as anchoring proteins or scaffolding components throughout the cell.

Materials and Methods

Cell Culture

NHBE cells were grown and differentiated as previously reported (408). Briefly, cells were expanded in bronchial epithelial cell growth medium [BEGM + SingleQuots containing 1.4 μM HC] and plated at low density on Snapwell polyester membranes and maintained under liquid-liquid interface growth conditions with complete BEGM until cells reached confluence (day 0). HC (1.4 μM) was withdrawn from HC0 cells at day 0. RA (500 nM) was added to BEGM for 48 h to promote differentiation (1). Air-liquid interface (ALI) culture conditions were initiated at day 2 by removal of apical medium, and basolateral growth medium was replaced with differentiation medium (DMEM/Ham's F12 + SingleQuots) containing 100 nM RA. All cells were grown at 37°C in a humidified CO_2 atmosphere. Control and HC0 NHBE cells formed pseudostratified epithelial tissues with ciliated and secretory surface cells. The mean transepithelial resistance (TER) was >1000 $\Omega \cdot cm^2$ for all epithelia after 1 week of ALI culture.

Quantitative RT-PCR

RNA was isolated using the Rneasy Mini Kit from Qiagen. cDNA was produced with the QuantiTect Reverse Transcription Kit with gDNA Wipeout also from Qiagen. PCR primers were designed using Primer3 and CLC sequence viewer software and ordered through the University of Minnesota BioMedical Genomics Center. Taqman probes were purchased through Life Technologies. Quantitative PCR amplification was performed on an Applied Biosystems 7300 Real-Time PCR system. Baseline and threshold values were set according to manufacturer's instructions. Relative expression

was quantified using the comparative cycle threshold ($2^{-\Delta Ct}$) method. GAPDH expression was used as the reference gene.

Immunofluorescence

Cells were grown on Snapwell polyester membranes as previously described and fixed in 4% PFA for 20 min. Cell membranes were permeated using 0.3% Triton-X and blocked with a PBS + 3% BSA solution for at least 1 hr. Cells were incubated with the primary antibody overnight at 4°C diluted in PBS + 3% BSA. Following incubation, cells were rinsed three times in PBS and incubated with the secondary antibody for 1 hr at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Monolayers were excised and mounted on microscope slides using VECATSHIELD HardSet mounting media (Vector Laboratories). Images were captured by an Olympus FV1000 Confocal Microscope. Primary antibodies for claudins were purchased from Santa Cruz Biotechnology (Dallas, TX; claudin-1 (sc-17658), claudin-3 (sc-17662), claudin-4 (sc-376643), claudin-8 (sc-66834), claudin-9 (sc398836), claudin-10 (sc-25710)) and secondary antibodies were purchased from Invitrogen (Alexa Fluor 488, 568, 647).

Dilution Potentials

Well differentiated NHBE cells were mounted in Ussing Chambers and bathed on apical and basolateral surfaces with standard saline solution containing containing (in mM) 130 NaCl, 6 KCl, 1.5 CaCl2, 1 MgCl2, 20 NaHCO3, 0.3 NaH2PO4, and 1.3 Na2HPO4, pH 7.4, and maintained at 37°C with 95% O2-5% CO2. Monolayers were voltage clamped and short-circuit current (I_{SC}) was measured. After a steady baseline had been achieved apical fluid was aspirated and replaced with modified saline solution containing 65 mM NaCl and 130 mM mannitol. Dilution potentials (ΔV) were measured immediately before and after apical fluid replacement. Calculation of permeability ratios were performed exactly as described in Hou et al. 2005 (147). Briefly, the ion permeability ratio (η) was calculated from the dilution potential using the Goldman-Hodgkin-Katz equation:

$$\eta = -(\epsilon - e^{v})/(1 - \epsilon e^{v}),$$

where η is the permeability ratio of Na^+ to Cl^- (P_{Na}/P_{Cl}); ϵ is the dilution factor (C_{basal}/C_{apical}); $v = eV/kT$ (V is the dilution potential, k is the Boltzmann constant, e is the elementary charge and T is temperature in Kelvin). The absolute permeabilities of Na^+ and Cl^- were calculated by the Kimizuka-Koketsu equation:

$$P_{Na} = (G/C) \cdot (RT/F^2) \cdot \eta/(1 + \eta)$$
$$P_{Cl} = (G/C) \cdot (RT/F^2)/(1 + \eta),$$

where C is the concentration in the diluted solution, R is the gas constant and F is the Faraday constant.

Statistics

All values reported are expressed as the mean ± SEM. For the qRT-PCR experiments reported in figure 1, an asterisk (*) represents significant differences between day zero and each subsequent day of differentiation as determined by an ANOVA followed by Dunnett's test for comparisons with a common control within each treatment condition. The diamonds (◊) represent significant differences between the HC0 condition compared to the corresponding day of differentiation in the control group as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test. In figure 2, statistical significance was determined by ANOVA followed by a Tukey-Kramer multiple comparisons test. A value of $p<0.05$ was considered significant.

Results

Quantitative RT-PCR was used to determine expression of 20 different claudin isoforms in control and HC0 cells. mRNA was collected and analyzed for days 0, 4, 8 and 24. NHBE cells express at least 10 claudin isoforms (Fig 1a, j) and the most highly expressed claudin transcripts were CLDN1, CLDN3, CLDN4 and CLDN7. Transcription

of certain claudins genes exhibited dynamic changes during differentiation. For example, CLDN9 expression was significantly increased at day 24 compared to day 0 in controls and was 16 fold greater compared to day 24 HC0 epithelia, which did not show time-dependent up-regulation (Fig 1f). CLDN3 expression also increased during differentiation in both control and HC0 epithelia (Fig 1b). CLDN7 transcription increased from day 0 to day 4 but remained steady in control and HC0 cells after day 4, although significantly reduced at days 4 and 8 in HC0 cells compared to control (Fig 1d). A similar pattern was observed for CLDN4, CLDN8, CLDN15 and CLDN23. CLDN10 expression was significantly reduced in day 24 HC0 cells compared to control (Fig 1g). HC0 cells consistently expressed reduced amounts of claudin mRNAs compared to control, but the most striking differences were observed for CLDN9 and CLDN10. Differentiated NHBE cells did not express detectable levels (>35 C_T) of at least 10 claudin isoforms, including claudin-2 and claudin-14 (Fig 1k). TJs are composed of membrane spanning proteins besides claudins, including occludin (OCLN) and junctional adhesion molecule 1 (JAM1). Transcription of OCLN mRNA was significantly reduced at all time-points in HC0 epithelia compared to control (Fig 1l). JAM1 mRNA expression in HC0 cells was less than control at day 8 and day 24. (Fig 1m).

Differentiated epithelia were mounted in Ussing chambers, then voltage clamped and exposed to benzamil (5 μM) and $CFTR_{inh}$-172 (20 μM) to inhibit basal ENaC and CFTR mediated ion transport. Mucosal saline solution was rapidly removed and replaced with an isosmolar solution containing only 60mM NaCl, which produced an apical-to-basal [NaCl] gradient across the epithelium and a diffusion potential of -1.69±0.5 and -1.46±0.3 mV in control and HC0 cells, respectively (mean±SEM). Using the Goldman-Hodgkin-Katz equation, we calculated the (P_{Na}/P_{Cl}) permeability ratio and plotted them for each condition in Fig 2c. The absolute permeabilities for Na^+ and Cl^- (P_{Na}, P_{Cl}) were calculated using the Kimizuka-Koketsu equation. P_{Na} was significantly greater at day 8 and day 24 in HC0 epithelia compared to control (Fig 2a). P_{Na} and P_{Cl} were reduced at day 24 compared to day 8 in control cells (Fig 2 a, b). P_{Cl} increased at day 24 compared to day 8 in HC0 cells but P_{Na}/P_{Cl} was not statistically different in day 24 control and HC0 epithelia. P_{Na}/P_{Cl} was greater than 1 in all days in both groups, indicating greater Na^+

selectivity compared to Cl^-. P_{Na}/P_{Cl} was significantly reduced at day 24 in control and HC0 cells. Finally, transepithelial conductance was measured and found to be greater in HC0 cells at day 24 compared to day 8 and compared to control at days 8 and 24.

Immunocytochemistry revealed the localization of claudins-1, -8, and -9 in the cilia, as well as the apical surface of differentiated NHBE cells (Fig 3). A similar localization pattern was observed for claudins-3 and -10 (Fig 4). Claudin-4, however, only localized at the apical surface and along the apical-basolateral junction. In day 4 NHBE cells, claudin-4 localization along cellular junctions was more apparent than day 24 (Fig 5a). In contrast, claudin-3 and claudin-10 did not localize along the junctional border in day 4 cells. Claudin-3 was observed to co-localize with acetylated α-tubulin, which is concentrated within cilia, indicating the distribution of claudin-3 within the cilia of differentiated surface cells (Fig 5b). Additionally, claudins-8 and -9 co-localize with $P2Y_2$ receptors in cilia (Fig 6a). Zona occludens-1 (ZO-1), a TJ scaffolding protein with multiple PDZ binding domains (129), displayed punctate localization at the level of the cilia and co-localized at some locations with acetylated α-tubulin (Fig 6b). ZO-1 also localized along the apical-basolateral junction and formed concentric rings around surface cells in differentiated epithelia (Fig 6b). These results demonstrated novel localization patterns for claudin and ZO-1 at cellular sites besides TJs.

Discussion

Claudin proteins determine the charge and size selectivity of the paracellular pathway (133, 318). The diversity of the claudin family of proteins allows for unique and tissue specific permeability characteristics of epithelial and endothelial tight junctions (TJs). In the present study, we investigated the effects of glucocorticoids on claudin expression and paracellular permeability during differentiation of normal human bronchial epithelial (NHBE) cells. NHBE cells withdrawn from supplementary hydrocortisone (HC0) did not have qualitatively different claudin expression profile compared to controls but did have significantly different paracellular permeability characteristics. A previous study on claudin expression in differentiated NHBE cells reported expression of claudin -1, -3, -4, -7, -10b, and -16 mRNAs with no detection of

claudins -2 and -10a (102). Results from the present study were consistent with this previous report, except for the expression of claudin-16. It was not possible to distinguish between claudin-10a and -10b with the RT-PCR technique employed in this study, although it is worth pointing out that claudin-10a appears to be restricted to the kidney (122, 376) and therefore NHBE cells presumably express claudin-10b. Additionally, CLDN8, CLDN9, CLDN12, CLDN15 and CLDN23 were also expressed in differentiated NHBE epithelia. HC appeared to have a limited effect on claudin transcription in that every claudin isoform detected by qRT-PCR in control cells was also expressed in HC0 cells. This suggested that the claudin expression profile in differentiated NHBE cells was established before mucociliary differentiation and was not altered by withdrawal of HC at the beginning of the differentiation protocol. Nevertheless, HC0 epithelia expressed markedly less CLDN9 and CLDN10 at day 24. A recent investigation found that glucocorticoids, but not mineralocorticoids, increased CLDN8 expression in primary tracheal epithelial cells, which correlated with increased TJ Cl^- permeability (176). In contrast, no effect of HC on CLDN8 transcription was observed in differentiated NHBE epithelia. Experiments performed using Caco-2 intestinal epithelial cells demonstrated that glucocorticoid exposure reduced expression of CLDN2, similar to results obtained in the present study (101).

Other TJ proteins were also shown to be modulated by the removal of HC. For instance, occludin expression was significantly reduced at all time-points after day 0 in HC0 epithelia. Occludin is a transmembrane protein found in all epithelial and endothelial tight junctions (375). However, the exact role it plays in the formation of junctional complexes is unknown. OCLN null mice do not exhibit deficiencies in barrier function and appear to have structurally normal TJs (310). However, overexpression studies have suggested that occludin could increase transepithelial resistance and occludin-deficient mice do have physiologic abnormalities that affect multiple organ systems (11, 323). In the present study, HC0 epithelia exhibited a significantly greater transepithelial conductance, which corresponded with a decrease in OCLN transcription. Therefore it is possible that reduction of TER (or increase on transepithelial conductance) observed in HC0 cells was due to reduced occludin expression within the TJ complex.

Although a qualitative difference in the claudin expression profile between control and HC0 epithelia was not observed, paracellular permeability was significantly altered in HC0 cells. HC0 epithelia had greater Na^+ permeability at day 8 and 24 compared to control. Furthermore, P_{Na} was not different at day 8 and 24 in HC0 epithelia, indicative of relatively stable Na^+ permeability during the later stages of differentiation. Furthermore, P_{Na} was not changed in HC0 cells, but the P_{Na}/ P_{Cl} ratio decreased from day 8 to day 24, reflecting an increase in P_{Cl} observed at day 24 in HC0 epithelia. Since reduced transcription of claudins -9 and -10 was observed, it is possible that one or both of these claudins selectively regulated anion flux through the TJ. A missense mutation of claudin-9 in mice was previously shown to cause deafness due to increased perilymph [K^+], suggesting that claudin-9 acts to inhibit paracellular K^+ flux (251). Additionally, claudin-10b is a cation-permeable claudin when transfected into MDCK cells which leads to increased paracellular permeability for cations like Li^+, Na^+, and K^+ (122). These findings and results from earlier studies suggest that the paracellular environment in HC0 cells may be claudin-10b depleted at day 24 and therefore are less selective for cations than TJs containing claudin-10b.

Interestingly, immunocytochemistry data from this investigation demonstrated claudin localization outside of TJs. Claudins-1, -3, -8, -9 and -10 were identified within cilia of differentiated NHBE surface cells. Moreover, their appearance in cilia was punctate and discrete, in contrast to the continuous, diaphanous appearance of acetylated α-tubulin, which labels the internal tubulin structure of motile cilia. This morphologic difference suggests that claudins exist at specific sites within cilia structures, possibly anchoring various proteins to the actin-cytoskeleton. This possibility is supported by the similarly discrete and punctate appearance of ZO-1 also within the cilia. ZO-1 is known to couple claudins to the actin-cytoskeleton at TJs through PDZ binding regions (133), which may be employed in a similar manner with claudins at regions outside of TJs. Claudins have recently been recognized for having additional roles in the cell besides facilitating ion movement across TJs. The most prominently reported additional function of claudins involves their recruitment into biochemically unique membrane domains, such as glycolipid-enriched and tetraspanin-enriched micro domains (GEMs and TEMs,

respectively), where they play key roles in supporting extra-cellular signal transduction pathways (262, 359, 400). Therefore, it is possible that claudins are recruited to distinct membrane micro domains near to motile cilia in airway epithelia. The role of claudins at this localization requires more investigation but could potentially affect cilia structure and function in mucociliary clearance.

Figure 4.1

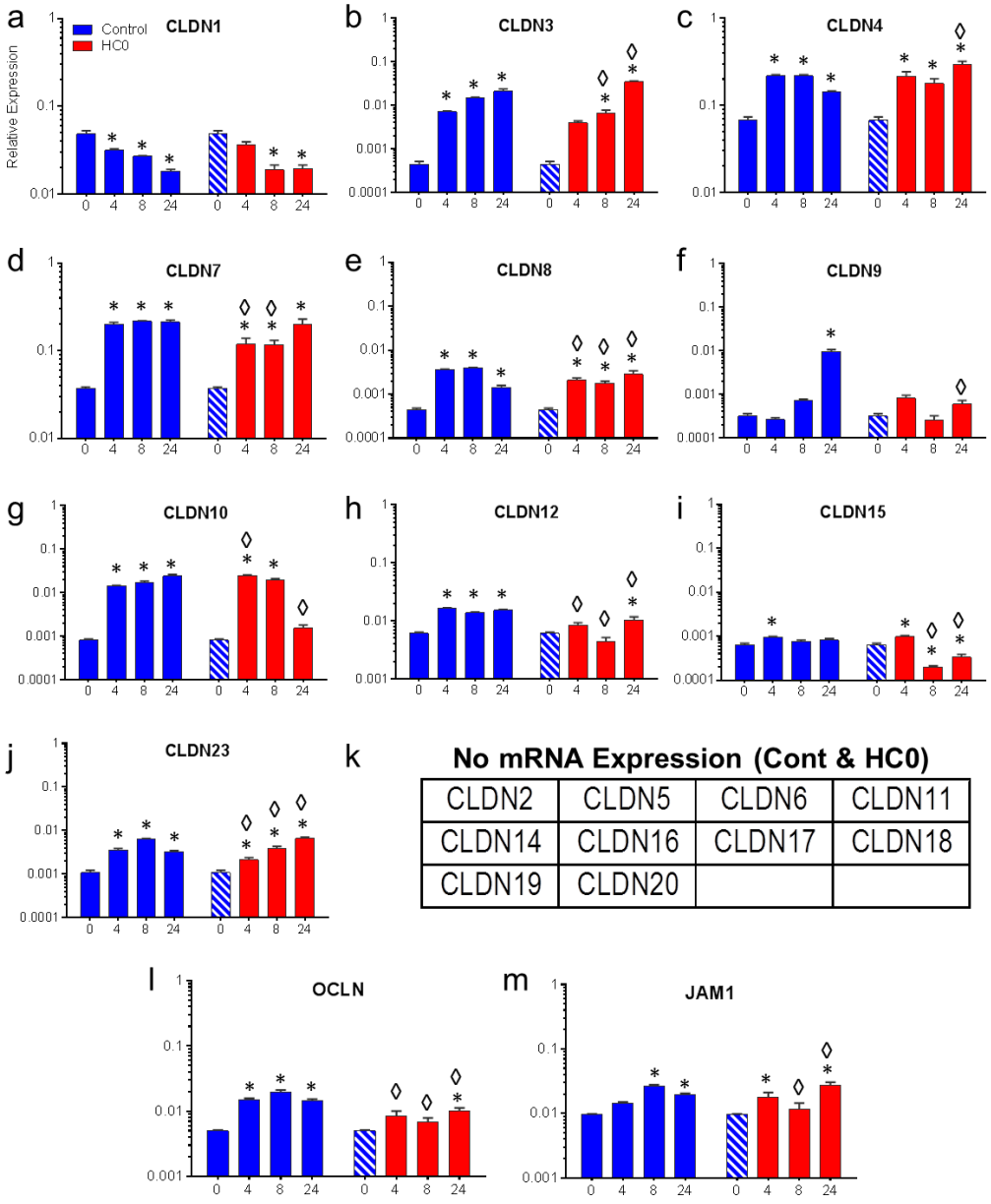

a
Control
HC0
Relative Expression
CLDN1
b
CLDN3
c
CLDN4
d
CLDN7
e
CLDN8
f
CLDN9
g
CLDN10
h
CLDN12
i
CLDN15
j
CLDN23
k
No mRNA Expression (Cont & HC0)
CLDN2 CLDN5 CLDN6 CLDN11
CLDN14 CLDN16 CLDN17 CLDN18
CLDN19 CLDN20
l
OCLN
m
JAM1

Figure 4.1. NHBE cells exhibit dynamic regulation of claudin mRNA expression during mucociliary differentiation. **A**: Quantitative RT-PCR analysis showing relative expression of claudin mRNA isoforms in comparison with GapDH (1.0), n=6 for each bar. Blue-and-white striped bars represent undifferentiated control data at day 0. **B**: Table listing claudin isoforms not detected by qRT-PCR. **C**: Expression of mRNAs for occludin and Jam1.

Figure 4.2

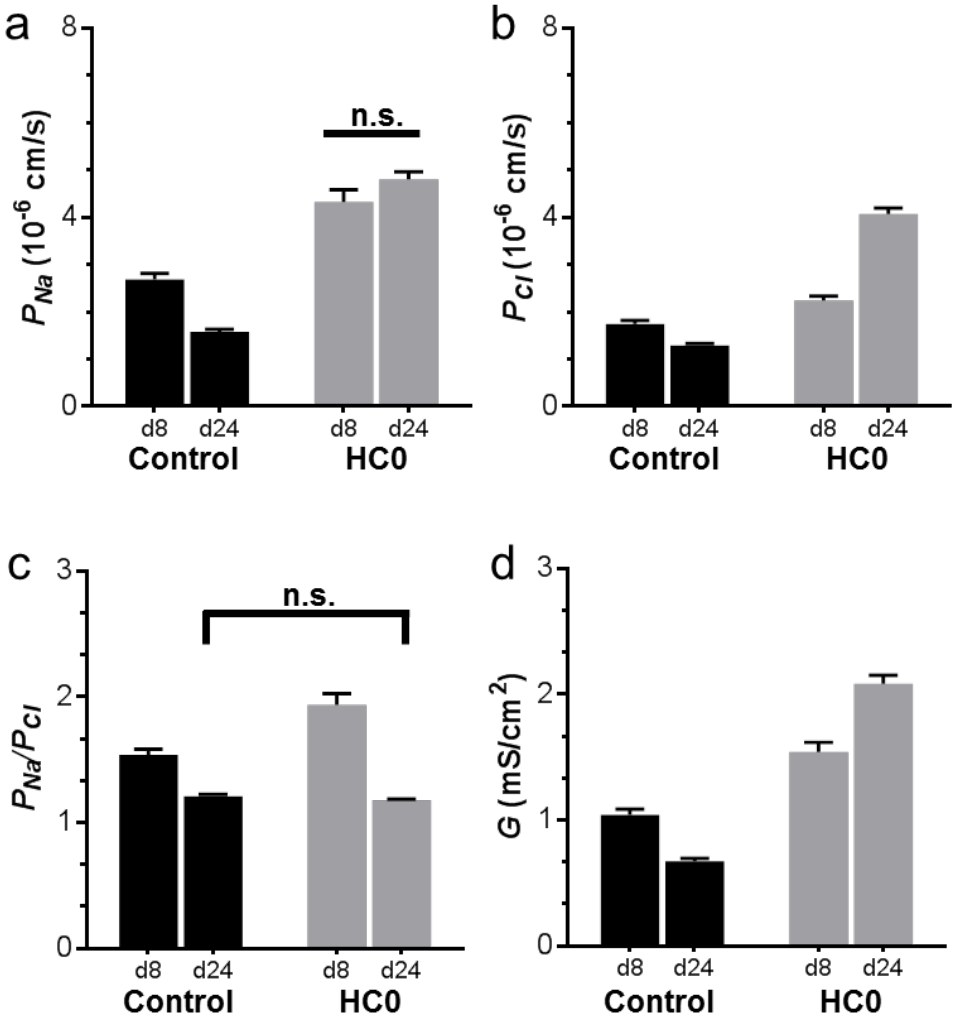


Figure 4.2. **Characterization of paracellular Na^+ and Cl^- permeability in control and HC0 NHBE epithelia** **A**: Na^+ permeability of day 8 and day 24 control and HC0 cells. **B**: Cl^- permeability of day 8 and day 24 control and HC0 cells. **C**: Ratio of P_{Na} to P_{Cl}. **D**: Conductance of day 8 and day 24 control and HC0 cells. *All bars in each histogram are significantly different from all other bars unless noted.

Figure 4.3

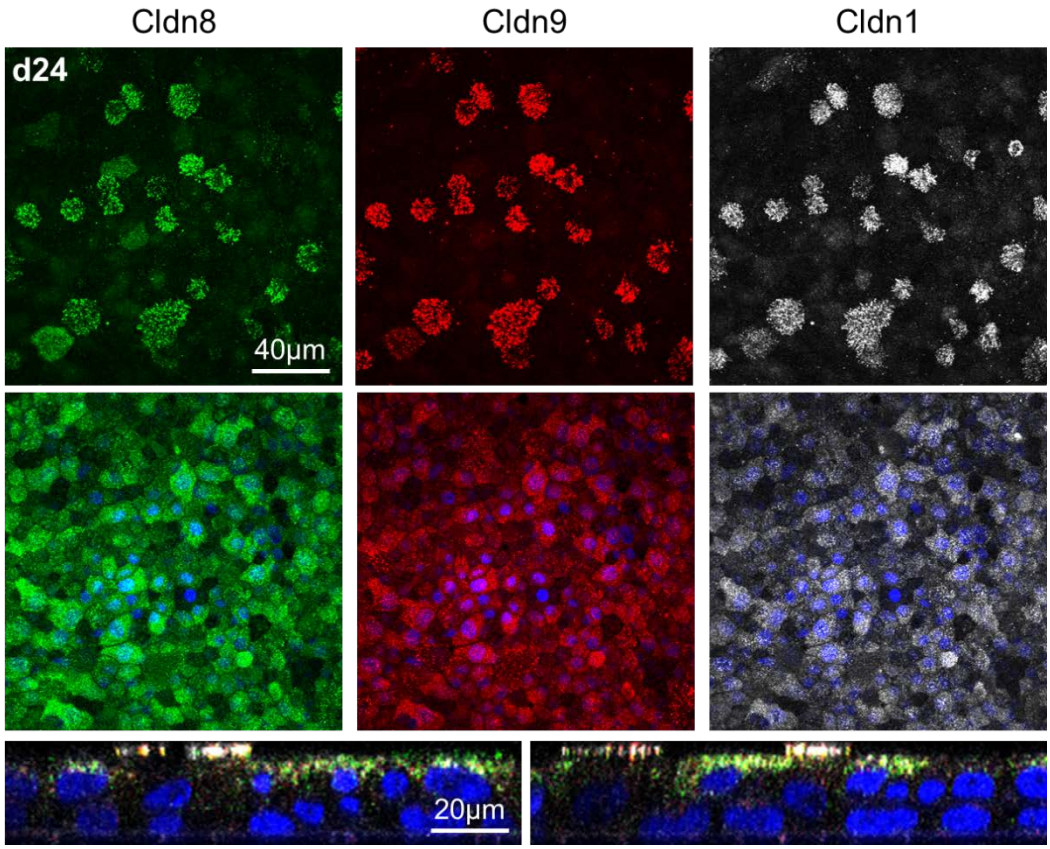


Figure 4.3. **Claudins localize at sites outside of tight junctions in differentiated NHBE cells.** Immunocytochemistry demonstrating claudin-8, claudin-9, and claudin-1 localization in cilia as well as in the apical membrane of differentiated surface cells. Transverse plane projections and cross sections of a representative control, day 24 epithelium.

Figure 4.4

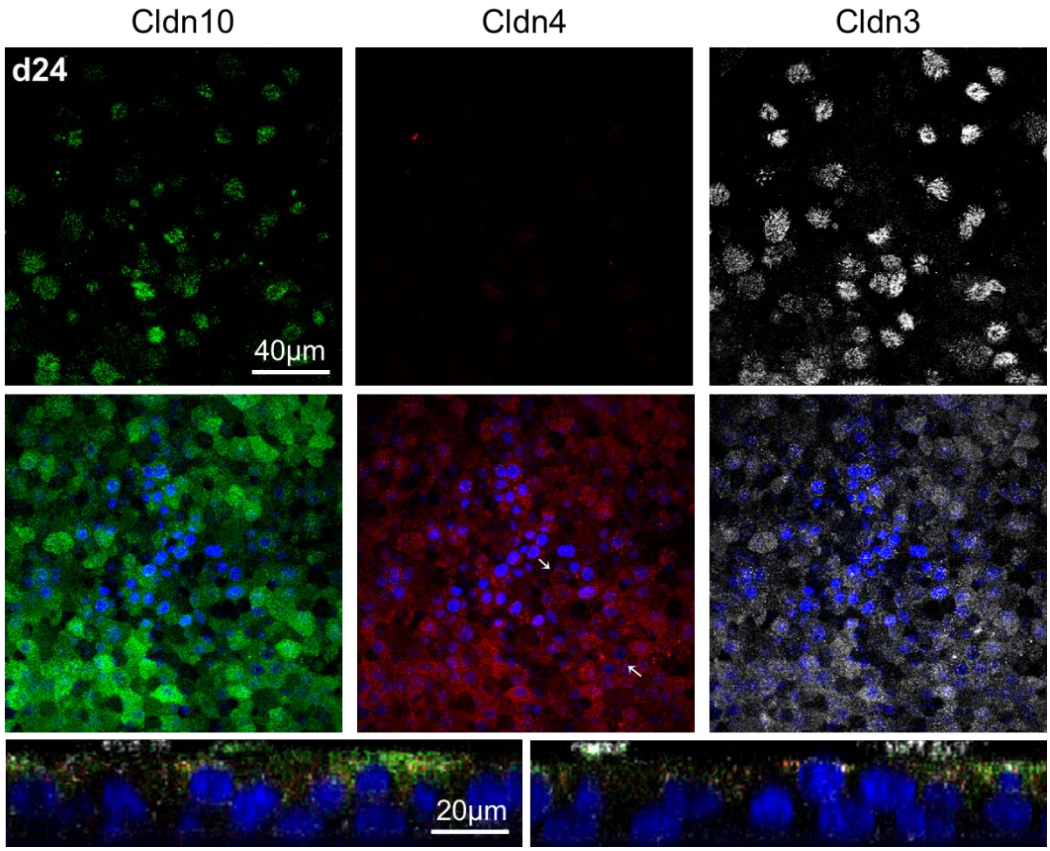


Figure 4.4. **Immunocytochemistry demonstrating claudin-3, -4 and claudin-10 localization in in differentiated NHBE cells.** Claudins -3 and -10 co-localize in cilia and the apical membrane. Claudin-4 only localizes at the apical membrane of surface cells. Transverse plane projections and cross sections are of a representative control, day 24 epithelium.

Figure 4.5

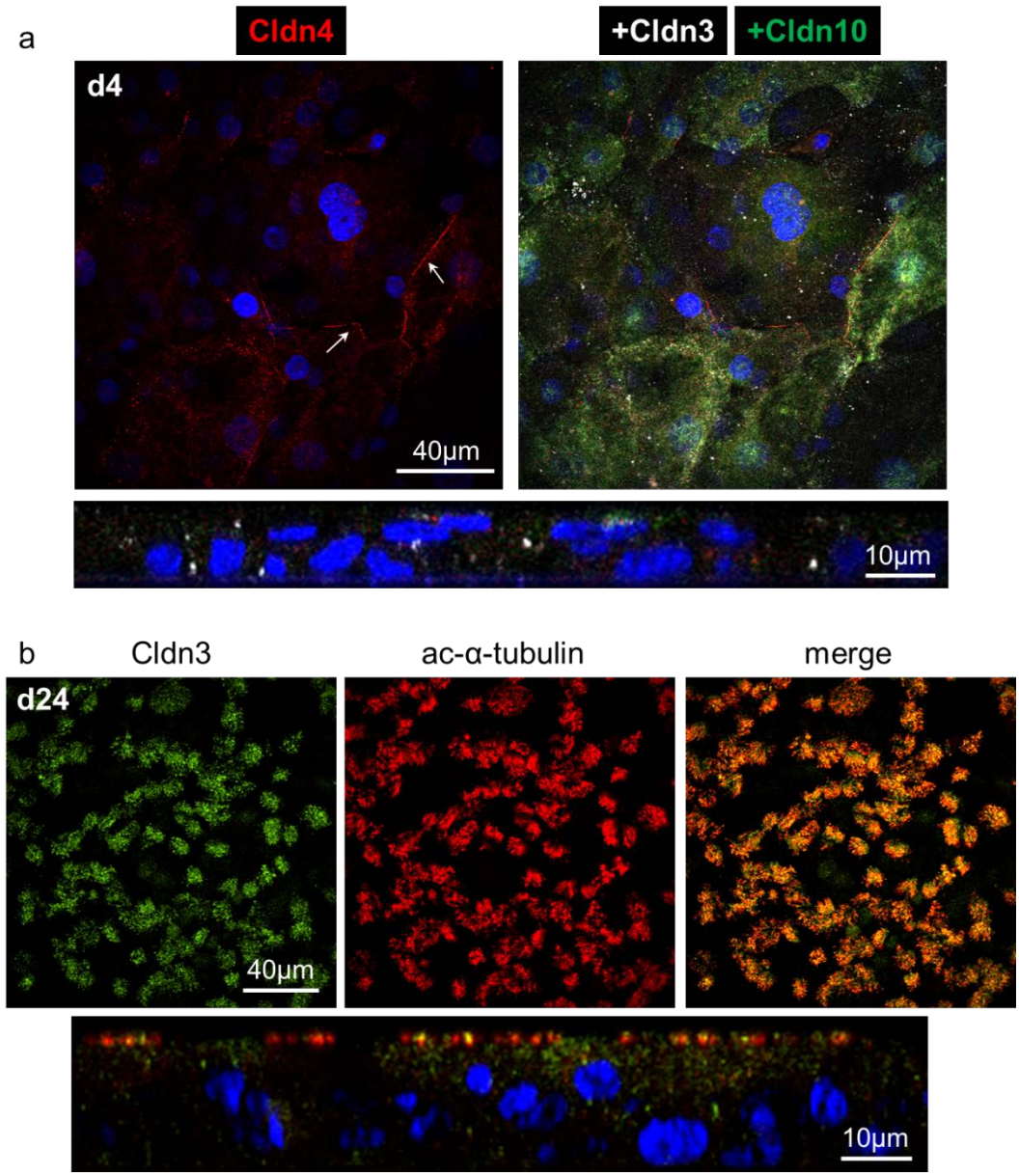


Figure 4.5. **Claudin-3 localizes in the cilia of differentiated NHBE surface cells. A**: Immunocytochemistry demonstrating claudin-4 localization along the lateral border of day 4 control cells. Claudins -3 and -10 do not co-localize with Claudin 4. **B**: Immunocytochemistry revealing co-localization of claudin-3 and acetylated- α-tubulin, labeling motile cilia in differentiated NHBE surface cells.

Figure 4.6

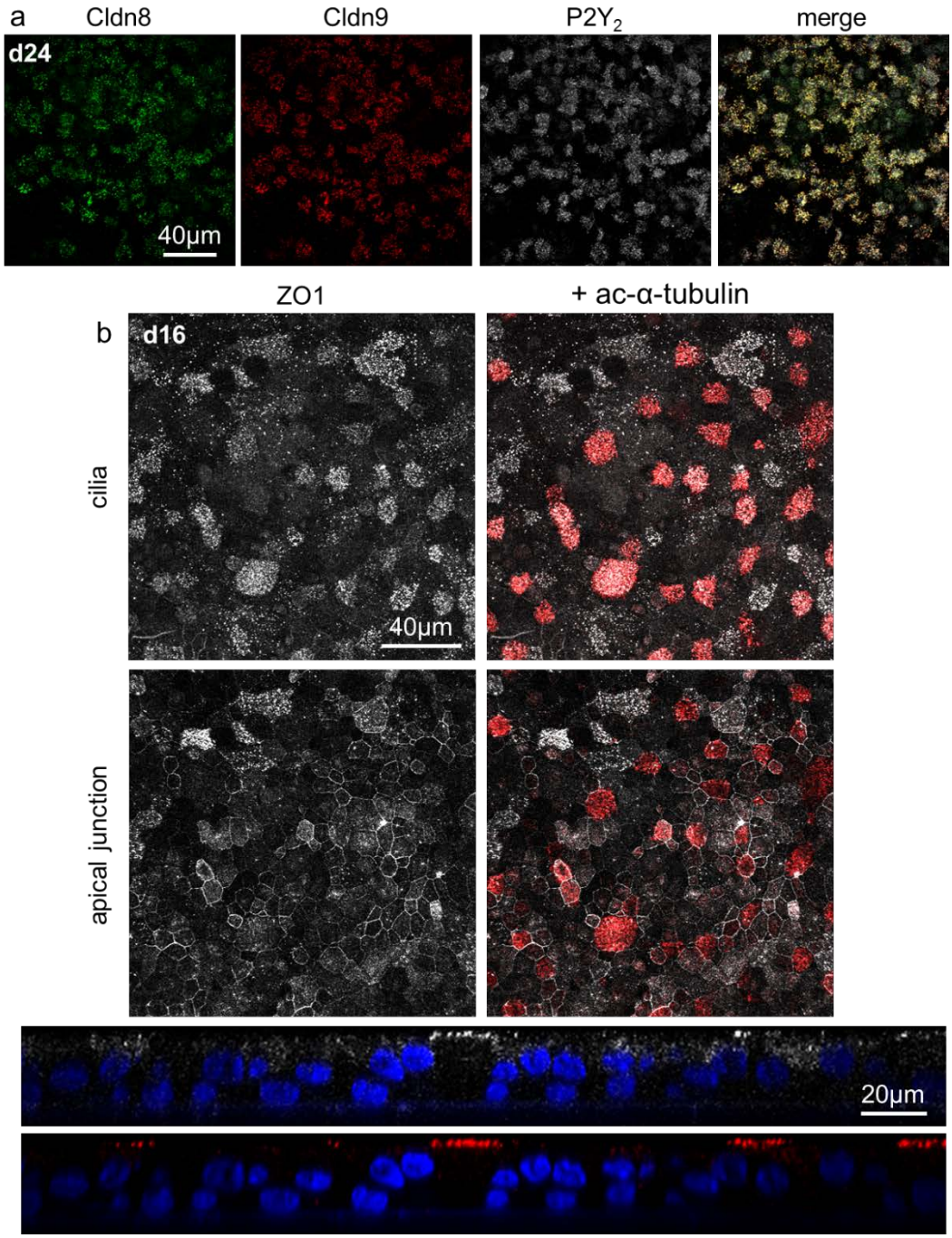

a
Cldn8
Cldn9
P2Y2
merge
d24
40μm
ZO1
+ ac-α-tubulin
b
d16
cilia
40μm
apical junction
20μm

Figure 4.6. **Claudins -3, -8 and -9, as well as $P2Y_2$ receptors, co-localize in the cilia of differentiated NHBE surface cells.** **A**: Immunocytochemistry showing co-localization of claudins -8, -9 and $P2Y_2$ in cilia at day 24 in control epithelia. **B**: ZO-1 localizes in cilia and the apical membrane of control surface cells at day 16. ZO-1 is also shown forming concentric rings around the basolateral membrane at tight junctions.

Chapter 5

Summary, Conclusions, Perspectives and Future Directions

Summary and Conclusions

Inhaled corticosteroids (ICS) are the standard of care for inflammatory airway diseases such as asthma, COPD and cystic fibrosis (CF). In these cases, ICS therapy vastly reduces the inflammation by limiting the production of pro-inflammatory cytokines and chemokines such as CXCR3, IL-1β, and IL-33 (118, 284). Corticosteroids are also known modulators of epithelial transport properties in the distal nephron and colon (315, 409). Moreover, *in vitro* studies of airway epithelial cell lines have demonstrated acute glucocorticoid regulation of epithelial sodium channels (ENaCs) by a similar mechanism as aldosterone in the aldosterone sensitive distal nephron (390). However, the effects of corticosteroids on electrolyte transport processes in a differentiated, pseudostratified airway epithelium in culture had not been examined. Therefore the objective of this thesis was to investigate the role of glucocorticoids, specifically hydrocortisone (HC), on the development and maintenance of the ion transport phenotype in differentiated normal human bronchial basal cells.

The first aim of this thesis was to investigate the role of hydrocortisone during mucociliary differentiation of airway basal cells and determine the effects of HC on surface cell differentiation. The second aim was to investigate the effects of HC on basal transepithelial ion transport processes and specific signaling pathways required for mucociliary clearance. The final aim was to characterize the paracellular transport pathway in differentiated epithelia and assess the effects of HC on paracellular ion permeability. The major conclusions from the experiments that were performed to address these aims are as follows:

1. *Hydrocortisone has a significant effect on transcription during NHBE differentiation.* Activated glucocorticoid receptors, expressed on the surface of the cell, are internalized and translocate to the nucleus where they exert transcriptional regulation on an array of genes. HC enhanced transcription of all three ENaC subunit mRNAs, consistent with previous reports. Additionally, HC exposure significantly increased mRNAs for β_2-AR, ENaC processing enzymes, BKα, $P2Y_4R$, claudin-9, as well as secretory cell markers MUC5B and SCGB1A1. Though HC withdrawal did not impede mucociliary differentiation,

HC0 epithelia expressed fewer secretory cell mRNAs and had qualitatively shorter cilia. Together, these results suggest HC supports transcriptional processes and differentiation pathways that are critical to mucociliary clearance.

2. *Hydrocortisone improves ion transport processes that are essential to mucociliary clearance.* Specifically, HC exposure increased benzamil-sensitive Na^+ absorption, was required for paxiline-sensitive K^+ secretion and enhanced salbutamol stimulated CFTR activity. While corticosteroids have previously been shown to regulate transcription of key ion channels in airway epithelial cells, the present studies have expanded our understanding of the physiologic significance of HC on ion transport in pulmonary epithelium. This is especially evident in the regulation of ENaC-dependent Na^+ absorption where much of the HC-induced increase in Na^+ transport was shown to be associated with enhanced trafficking of ENaC subunits into the apical membrane and cilia of surface epithelial cells. Furthermore, HC was shown to be absolutely required for BK channel function, even though expression of the channel, its regulatory subunits and relevant receptor signaling mechanisms were still present in the apical membrane of HC deficient epithelia.
3. *Hydrocortisone transcriptional regulation has direct and significant effects on ion transport processes.* HC withdrawal caused an increase in transcription of KCNMA1 mRNAs that contain the STREX exon, a splice-variant of BKα that is subject to PKA-mediated channel inhibition, which resulted in the complete loss of BK activation after purinergic receptor activation. This result emphasizes the functional, downstream consequences of corticosteroid-regulated transcription in airway epithelial cells. Moreover, this result demonstrates the influence of a single variable (HC) on a physiologic process. While redundancy and graded effects are apparent in nearly all biologic systems, here the absence of just one factor revealed a binary outcome in differentiated NHBE cells.
4. *Hydrocortisone decreases ion permeability characteristics of tight junctions, possibly through transcriptional regulation of claudin family proteins.* HC

reduced both Na^+ and Cl^- permeability through the paracellular space, demonstrating a role for HC in the maintenance of epithelial TJs.

Perspectives and Future Studies

Previous studies on the effects of corticosteroids on airway epithelial cells were limited to *in vitro* systems that do not recapitulate the anatomical and physiological properties of native pulmonary epithelium (160, 205, 313). The differentiation protocol employed in the present work transforms basal airway epithelial cells into pseudostratified epithelia with ciliated and secretory surface cells possessing physiologically responsive ion transport pathways. The advantage of studying these authentic representations of the airway epithelium is the capacity to compare and translate *in vitro* results to native tissues and allows investigators a unique opportunity to examine native airway epithelial physiology in a controlled environment without confounding contributions from the mesenchyme, nervous system or adaptive immune system. In the present work we demonstrated the ability to study ion transport and signaling processes that are critical to normal mucociliary clearance in differentiated epithelia. Additionally, we strategically manipulated the *in vitro* differentiation conditions to test hypotheses about the development of these critical physiologic pathways. Previous work has demonstrated HC regulation of ENaC in airway epithelial cells, however the epithelia formed by these cells do not exhibit the overall structural features or cellular diversity of the native epithelium. Here, we have shown the effects of HC on the mucociliary differentiation of airway basal cells and evaluated the global ion transport phenotype of these functional epithelia.

These lab derived epithelia are useful tools for studying epithelial properties and physiologic processes that are relevant to the pulmonary epithelium. One such process is mucociliary clearance (MCC), which is the regulated removal of inhaled particles and debris from the airways through the coordinated beating of surface cell cilia. Ion transport by airway epithelium provides the osmotic driving forces necessary to sustain periciliary fluid (PCL) depth and effective MCC. Although the studies included in the present work demonstrate our ability to study transepithelial ion transport, differentiated epithelia are well suited for investigations of general MCC. Since the differentiation process produces pseudostratified epithelium with surface secretory and ciliated cells, these *in vitro* derived tissues are equipped with the necessary components to both secrete

and propel mucus. Evaluation of MCC can be achieved optically by tracking the movement of mucus rafts or through fluorescent beads released on the surface of the epithelia (224). Addition of pharmacologic compounds that increase or decrease ciliary beat frequency (CBF) can be used to study the biophysics and velocity of MCC in normal cells. Furthermore, the viscosity of the PCL can be manipulated by the addition of non-isotonic solutions or by the addition of mucolytics which disrupt the mucus hydrogel (406). A variety of variables can be tuned and manipulated on these epithelia to further our understanding of MCC. The results of such studies can be compared to *in vivo* measurements made in healthy individuals as well as patients with COPD or CF. Such comparisons could be helpful in predicting what component of the mucociliary apparatus is causing reduced MCC in pathologic states and lead to patient specific treatment options.

Personalized medicine is the idea of tailored intervention and treatment strategies based on individual patient needs and responses. Harvesting patient airway basal cells to produce differentiated epithelia would allow physicians to predict the best treatment strategies for individuals suffering from inflammatory airway diseases. Combined ICS and long-acting β-agonist (LABAs) therapy is the current standard of care for asthma despite the heterogeneity in therapeutic response observed in patients. Additionally, a subset of asthma patients do not respond to ICS therapy and are designated as being corticosteroid insensitive. Several molecular mechanisms have been described to account for corticosteroid resistance, including aberrant ERK, MAPK, and JNK activity, variant glucocorticoid receptor (GR) expression and reduced expression of histone deacetylase 2 (HDAC2) (19). Laboratory derived, patient specific airway epithelia could be used to determine the precise mechanism of corticosteroid resistance in an individual or more broadly be used to screen for the best therapeutic approach for a given asthma patient. Additionally, healthy epithelia can be treated with a selective glucocorticoid antagonist (e.g. RU40555) so that the differentiated epithelium can be employed to screen for alternative and possibly more effective anti-inflammatory drugs to treat corticosteroid insensitive asthma. Furthermore, corticosteroid resistant epithelia undergo distinct remodeling and have characteristics of squamous metaplasia that are susceptible to

epithelial sloughing (116). *In vitro* differentiated epithelia could be used to study the morphologic changes to pseudostratified tissues that occur in pathologic states and assist in our understanding of epithelial remodeling.

Another patient specific application for these laboratory derived tissues is the translation of differentiated epithelia from *in vitro* to clinically relevant implantations. Pseudostratified epithelia that are physiologically responsive form just eight days after the initiation of differentiation. Differentiated epithelia grown from patient-specific airway basal cells can be harvested and seeded onto engineered airway scaffolds and transplanted back into individual patients. In this way, complications due to host immune responses and transplant rejection can be avoided. Moreover, epithelia can be pre-screened in the laboratory to assess physiologic responsiveness before they are implanted. Similarly, nasal epithelial cells or basal cells from other absorptive epithelia, such as colon, could be harvested from patients to form implantable epithelia if their pulmonary tissue is severely compromised. Gene editing techniques, such as CRISPR/Cas or TALEN systems, could also be employed to produce a healthy tissue that no longer contains, for example, a CFTR mutation or an unresponsive isoform of the glucocorticoid receptor.

The use of laboratory derived epithelia as a drug screening platform is in itself an intriguing prospect. Besides asthma, many large airway diseases are characterized by reduced or dysfunctional MCC. In cystic fibrosis, loss of anion secretion through CFTR and an increase in ENaC mediated Na^+ absorption leads to a decrease in PCL height, mucus stasis and chronic infection. CF is a debilitating disease in which patient care centers on symptom management, not cures. Current treatment strategies for the common ΔF508 mutation have focused on corrector or chaperone pharmaceuticals that promote maturation of defunct CFTR channels (303). Despite initial optimism, clinical outcomes with these drugs have been nominal and do not reverse disease progression. One severe shortcoming in the drug design process is that molecules are screened purely on the basis of restoration of CFTR channel conductance. While this is an obvious strategy it does not take into account the role CFTR plays within the greater cellular environment and the larger issue of MCC in CF. Rather than assessing CFTR activity

directly in Ussing chambers, differentiated epithelia can be used as a drug discovery platform targeting an increase in MCC as well as restored CFTR channel function. Basal airway epithelial cells containing the ΔF508 mutation were shown to form differentiated and psuedostratified epithelia in Chapter 2 of this thesis. Expanding on these studies, a library of molecules can be screened for their ability to increase MCC by direct measurement of CBF or PCL depth. CBF is directly correlated with PCL volume and MCC, and can be determined with optical tracing techniques (224). Additionally, PCL height can be determined by direct measurement of Texas-red stained fluid and colorimetric assays can be designed to assess dilution of Texas-red intensity (203). Automated image acquisition and signal processing would enable high throughput screening of CF epithelia MCC and broader assessment of the physiologic effects of specific drugs. The advantage of this approach is that it may uncover novel molecular processes that contribute to CF pathology. For example, in the present study, CFTR was shown to co-localize with β_2-ARs within cilia of differentiated surface cells. β_2-AR activation results in increased cellular cAMP that not only stimulates CFTR but also leads to an increase of CBF (316). Identification of a compound that restores MCC in CF epithelia may uncover unknown associations between CFTR and β_2-ARs that are relevant to CBF and independent of CFTR channel activity.

A consistent result throughout these studies was the distinct cellular localization of several ion channels and receptors within the motile cilia of differentiated airway surface cells. Previous studies have demonstrated cilia localization of specific ion channels, but here we demonstrate consistent utilization of the cilia membrane domain for unique and overlapping signaling regions on airway surface cells. Although we observed this phenomenon, we did not investigate the processes involved in trafficking and anchoring of specific proteins within cilia. An interesting result presented in Chapter 4 of this thesis involved the localization of ZO-1 and certain claudin proteins to cilia. The presence of ZO-1, a well-characterized anchoring protein, suggests a highly organized structure that links and supports the core proteins of motile cilia with cytosolic microfilaments. Furthermore, claudins have been shown to exist in chemically unique membrane domains, and their inclusion within cilia suggests there may be a unique

chemical composition of cilia phospholipids that preferentially support the function of specific ion channels and signaling receptors. The recruitment of specific proteins and partitioning of membrane phospholipids has been well studied in primary cilium (90, 149, 247, 355). Though distinct from motile cilia, primary cilia are rod-like membrane projections that extend from the surface of many cell types and are thought to serve as sensory antenna that detects and transmits chemical and mechanical cues from the external environment. Like motile cilia on airway surface cells, the primary cilia membrane is contiguous with the plasma membrane and studies have demonstrated chemically distinct lipid composition in primary cilia membranes. Furthermore, lipid-raft associated proteins are similarly targeted to the ciliary membrane. Protein delivery into primary cilia is believed to occur by targeted accumulation of vesicles at the base of the cilia and these proteins are retained in the ciliary domain through a 'necklace' that forms a diffusion barrier for entry and exit from the cilium (51), similar to the fence function of epithelial TJs. It is possible that we have observed a similar diffusion barrier structure at the base of motile cilia in differentiated NHBE that is being linked with the actin-cytoskeleton through ZO-1 and claudin interactions.

Motile cilia serve two primary functions: 1) propel the mucus hydrogel out of the airways towards the pharynx, and 2) produce convection that mixes the molecular milieu of the periciliary fluid. Stirring of the PCL is critical for rapidly distributing secreted material. Moreover, solute permeation and water movement across planar lipid-bilayers are subject to reduced diffusion caused by solute accumulation at the unstirred layer (USL) (230, 248). Thus, ciliary beating is necessary for airway hydration to be effective. Localization of ion channels within motile cilia reduces ion accumulation within the USL and helps to sustain the chemical driving force for diffusion (both influx and efflux) through open channel pores. Furthermore, ciliated surface cells have much greater surface area than non-ciliated cells, which allows for an increase in the number of channels and enhanced ion conductance, facilitating a greater electrolyte flux across the apical membrane. Future experiments that investigate the capacitance of ciliary membranes would further reveal the biophysics dictating ion transport through these unique structures. Myelin sheaths along neuronal axons decrease the capacitance of the

membrane which allows for propagation of relatively small signals, sometimes carried by a small number of ions (345). Due to their low cytosolic volume, cilia may be particularly responsive to very small changes in ion uptake or efflux that could potentially regulate channel activity or affect the driving force for ion movement across the membrane.

Beyond ion channels, receptors were also shown to localize within cilia of differentiated NHBE cells. $P2Y_2$ receptors were found in both cilia and the apical membrane of surface cells, while $P2Y_4$ receptors were only detected at the apical membrane. These unique and overlapping signaling regions may play significant roles in airway innate defense. The PCL can be thought of as a buffer zone that limits direct epithelial interactions with inhaled particles. Activation of cilia-bound receptors is indicative of signals arising from the PCL bulk fluid or from entrapped debris in the mucus hydrogel. Apical surface bound receptors are likely stimulated only after cilia-bound receptors have been activated and therefore are indicative of deeper infiltration through the PCL. Secretory surface cells, which release mucins as well as pro-inflammatory cytokines and chemokines, do not have cilia and therefore surface receptors are only activated when danger signals reach the apical membrane of luminal surface cells. In this way, differential purinergic signaling may provide for a graded response that may be necessary to combat invasive insults in an appropriate manner.

References

1. **Adler KB, Tuvim MJ, and Dickey BF**. Regulated mucin secretion from airway epithelial cells. *Front Endocrinol (Lausanne)* 4: 129, 2013.
2. **Aksoy MO, Mardini IA, Yang Y, Bin W, Zhou S, and Kelsen SG**. Glucocorticoid effects on the beta-adrenergic receptor-adenylyl cyclase system of human airway epithelium. *J Allergy Clin Immunol* 109: 491-497, 2002.
3. **Aleksandrov L, Aleksandrov AA, Chang XB, and Riordan JR**. The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction, whereas the Second Is a Site of Rapid Turnover. *J Biol Chem* 277: 15419-15425, 2002.
4. **Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, and Fromm M**. Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. *J Cell Sci* 115: 4969-4976, 2002.
5. **Ambort D, Johansson ME, Gustafsson JK, Ermund A, and Hansson GC**. Perspectives on mucus properties and formation--lessons from the biochemical world. *Cold Spring Harb Perspect Med* 2: 2012.
6. **Anagnostopoulou P, Riederer B, Duerr J, Michel S, Binia A, Agrawal R, Liu X, Kalitzki K, Xiao F, Chen M, Schatterny J, Hartmann D, Thum T, Kabesch M, Soleimani M, Seidler U, and Mall MA**. SLC26A9-mediated chloride secretion prevents mucus obstruction in airway inflammation. *J Clin Invest* 122: 3629-3634, 2012.
7. **Andrini O, Keck M, Briones R, Lourdel S, Vargas-Poussou R, and Teulon J**. ClC-K chloride channels: emerging pathophysiology of Bartter syndrome type 3. *Am J Physiol Renal Physiol* 308: F1324-1334, 2015.
8. **Araya J, Cambier S, Markovics JA, Wolters P, Jablons D, Hill A, Finkbeiner W, Jones K, Broaddus VC, Sheppard D, Barzcak A, Xiao Y, Erle DJ, and Nishimura SL**. Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients. *J Clin Invest* 117: 3551-3562, 2007.
9. **Astrand AB, Hemmerling M, Root J, Wingren C, Pesic J, Johansson E, Garland AL, Ghosh A, and Tarran R**. Linking increased airway hydration, ciliary beating, and mucociliary clearance through ENaC inhibition. *Am J Physiol Lung Cell Mol Physiol* 308: L22-32, 2015.
10. **Baas AF, Kuipers J, van der Wel NN, Batlle E, Koerten HK, Peters PJ, and Clevers HC**. Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. *Cell* 116: 457-466, 2004.
11. **Balda MS, Flores-Maldonado C, Cereijido M, and Matter K**. Multiple domains of occludin are involved in the regulation of paracellular permeability. *J Cell Biochem* 78: 85-96, 2000.
12. **Balda MS, Gonzalez-Mariscal L, Contreras RG, Macias-Silva M, Torres-Marquez ME, Garcia-Sainz JA, and Cereijido M**. Assembly and sealing of tight junctions: possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J Membr Biol* 122: 193-202, 1991.
13. **Ballard PL, and Ballard RA**. Scientific basis and therapeutic regimens for use of antenatal glucocorticoids. *Am J Obstet Gynecol* 173: 254-262, 1995.

14. **Bangel N, Dahlhoff C, Sobczak K, Weber WM, and Kusche-Vihrog K**. Upregulated expression of ENaC in human CF nasal epithelium. *J Cyst Fibros* 7: 197-205, 2008.
15. **Bardou O, Trinh NT, and Brochiero E**. Molecular diversity and function of K+ channels in airway and alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 296: L145-155, 2009.
16. **Barnes PJ**. Corticosteroid effects on cell signalling. *Eur Respir J* 27: 413-426, 2006.
17. **Barnes PJ**. Drugs for asthma. *Br J Pharmacol* 147 Suppl 1: S297-303, 2006.
18. **Barnes PJ**. How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br J Pharmacol* 148: 245-254, 2006.
19. **Barnes PJ**. Therapeutic approaches to asthma-chronic obstructive pulmonary disease overlap syndromes. *J Allergy Clin Immunol* 136: 531-545, 2015.
20. **Baum B, and Georgiou M**. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol* 192: 907-917, 2011.
21. **Bear CE, and Reyes EF**. cAMP-activated chloride conductance in the colonic cell line, Caco-2. *Am J Physiol* 262: C251-256, 1992.
22. **Beasley R, Roche WR, Roberts JA, and Holgate ST**. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev Respir Dis* 139: 806-817, 1989.
23. **Beghe B, Rabe KF, and Fabbri LM**. Phosphodiesterase-4 inhibitor therapy for lung diseases. *Am J Respir Crit Care Med* 188: 271-278, 2013.
24. **Ben-Yosef T, Belyantseva IA, Saunders TL, Hughes ED, Kawamoto K, Van Itallie CM, Beyer LA, Halsey K, Gardner DJ, Wilcox ER, Rasmussen J, Anderson JM, Dolan DF, Forge A, Raphael Y, Camper SA, and Friedman TB**. Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration. *Hum Mol Genet* 12: 2049-2061, 2003.
25. **Benos DJ, Mandel LJ, and Balaban RS**. On the mechanism of the amiloride-sodium entry site interaction in anuran skin epithelia. *J Gen Physiol* 73: 307-326, 1979.
26. **Bentzen BH, Nardi A, Calloe K, Madsen LS, Olesen SP, and Grunnet M**. The small molecule NS11021 is a potent and specific activator of Ca2+-activated big-conductance K+ channels. *Mol Pharmacol* 72: 1033-1044, 2007.
27. **Berger WE, and Nadel JA**. Efficacy and safety of formoterol for the treatment of chronic obstructive pulmonary disease. *Respir Med* 102: 173-188, 2008.
28. **Bernard K, Bogliolo S, Soriani O, and Ehrenfeld J**. Modulation of calcium-dependent chloride secretion by basolateral SK4-like channels in a human bronchial cell line. *J Membr Biol* 196: 15-31, 2003.
29. **Bhalla V, and Hallows KR**. Mechanisms of ENaC regulation and clinical implications. *J Am Soc Nephrol* 19: 1845-1854, 2008.
30. **Bhowmik A, Chahal K, Austin G, and Chakravorty I**. Improving mucociliary clearance in chronic obstructive pulmonary disease. *Respir Med* 103: 496-502, 2009.
31. **Billington CK, Ojo OO, Penn RB, and Ito S**. cAMP regulation of airway smooth muscle function. *Pulm Pharmacol Ther* 26: 112-120, 2013.

32. **Blanchard A, Jeunemaitre X, Coudol P, Dechaux M, Froissart M, May A, Demontis R, Fournier A, Paillard M, and Houillier P**. Paracellin-1 is critical for magnesium and calcium reabsorption in the human thick ascending limb of Henle. *Kidney Int* 59: 2206-2215, 2001.
33. **Blouquit-Laye S, and Chinet T**. Ion and liquid transport across the bronchiolar epithelium. *Respir Physiol Neurobiol* 159: 278-282, 2007.
34. **Bohme I, and Beck-Sickinger AG**. Illuminating the life of GPCRs. *Cell Commun Signal* 7: 16, 2009.
35. **Boon M, De Boeck K, Jorissen M, and Meyts I**. Primary ciliary dyskinesia and humoral immunodeficiency--is there a missing link? *Respir Med* 108: 931-934, 2014.
36. **Boucher RC**. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* 58: 157-170, 2007.
37. **Boucher RC**. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. *Trends Mol Med* 13: 231-240, 2007.
38. **Boyd C, and Naray-Fejes-Toth A**. Steroid-mediated regulation of the epithelial sodium channel subunits in mammary epithelial cells. *Endocrinology* 148: 3958-3967, 2007.
39. **Brune K, Frank J, Schwingshackl A, Finigan J, and Sidhaye VK**. Pulmonary epithelial barrier function: some new players and mechanisms. *Am J Physiol Lung Cell Mol Physiol* 308: L731-745, 2015.
40. **Bruns JB, Carattino MD, Sheng S, Maarouf AB, Weisz OA, Pilewski JM, Hughey RP, and Kleyman TR**. Epithelial Na+ channels are fully activated by furin- and prostasin-dependent release of an inhibitory peptide from the gamma-subunit. *J Biol Chem* 282: 6153-6160, 2007.
41. **Burdyga T, and Wray S**. Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. *Nature* 436: 559-562, 2005.
42. **Burnstock G, Brouns I, Adriaensen D, and Timmermans JP**. Purinergic signaling in the airways. *Pharmacol Rev* 64: 834-868, 2012.
43. **Button B, Cai LH, Ehre C, Kesimer M, Hill DB, Sheehan JK, Boucher RC, and Rubinstein M**. A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. *Science* 337: 937-941, 2012.
44. **Camner P, Mossberg B, and Philipson K**. Tracheobronchial clearance and chronic obstructive lung disease. *Scand J Respir Dis* 54: 272-281, 1973.
45. **Carattino MD, Passero CJ, Steren CA, Maarouf AB, Pilewski JM, Myerburg MM, Hughey RP, and Kleyman TR**. Defining an inhibitory domain in the alpha-subunit of the epithelial sodium channel. *Am J Physiol Renal Physiol* 294: F47-52, 2008.
46. **Carattino MD, Sheng S, Bruns JB, Pilewski JM, Hughey RP, and Kleyman TR**. The epithelial Na+ channel is inhibited by a peptide derived from proteolytic processing of its alpha subunit. *J Biol Chem* 281: 18901-18907, 2006.
47. **Carballo E, Lai WS, and Blackshear PJ**. Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* 95: 1891-1899, 2000.
48. **Carballo E, Lai WS, and Blackshear PJ**. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science* 281: 1001-1005, 1998.

49. **Cazzola M, Page CP, Calzetta L, and Matera MG**. Pharmacology and therapeutics of bronchodilators. *Pharmacol Rev* 64: 450-504, 2012.
50. **Cereijido M, Robbins ES, Dolan WJ, Rotunno CA, and Sabatini DD**. Polarized monolayers formed by epithelial cells on a permeable and translucent support. *J Cell Biol* 77: 853-880, 1978.
51. **Chailley B, and Boisvieux-Ulrich E**. Detection of plasma membrane cholesterol by filipin during microvillogenesis and ciliogenesis in quail oviduct. *J Histochem Cytochem* 33: 1-10, 1985.
52. **Champigny G, Voilley N, Lingueglia E, Friend V, Barbry P, and Lazdunski M**. Regulation of expression of the lung amiloride-sensitive Na+ channel by steroid hormones. *EMBO J* 13: 2177-2181, 1994.
53. **Che P, Tang H, and Li Q**. The interaction between claudin-1 and dengue viral prM/M protein for its entry. *Virology* 446: 303-313, 2013.
54. **Chen R, Lim JH, Jono H, Gu XX, Kim YS, Basbaum CB, Murphy TF, and Li JD**. Nontypeable Haemophilus influenzae lipoprotein P6 induces MUC5AC mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways. *Biochem Biophys Res Commun* 324: 1087-1094, 2004.
55. **Chen Y, Zhao YH, and Wu R**. Differential regulation of airway mucin gene expression and mucin secretion by extracellular nucleotide triphosphates. *Am J Respir Cell Mol Biol* 25: 409-417, 2001.
56. **Cheng JB, Goldfien A, Ballard PL, and Roberts JM**. Glucocorticoids increase pulmonary beta-adrenergic receptors in fetal rabbit. *Endocrinology* 107: 1646-1648, 1980.
57. **Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, and Smith AE**. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 63: 827-834, 1990.
58. **Choi JY, Cho KN, Yoo KH, Shin JH, and Yoon JH**. Retinoic acid depletion induces keratinizing squamous differentiation in human middle ear epithelial cell cultures. *Acta Otolaryngol* 123: 466-470, 2003.
59. **Chopra DP**. Retinoid reversal of squamous metaplasia in organ cultures of tracheas derived from hamsters fed on vitamin A-deficient diet. *Eur J Cancer Clin Oncol* 19: 847-857, 1983.
60. **Clevers H**. Wnt/beta-catenin signaling in development and disease. *Cell* 127: 469-480, 2006.
61. **Cole AM, Ganz T, Liese AM, Burdick MD, Liu L, and Strieter RM**. Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* 167: 623-627, 2001.
62. **Collins S, Caron MG, and Lefkowitz RJ**. Beta-adrenergic receptors in hamster smooth muscle cells are transcriptionally regulated by glucocorticoids. *J Biol Chem* 263: 9067-9070, 1988.
63. **Colussi G, De Ferrari ME, Tedeschi S, Prandoni S, Syren ML, and Civati G**. Bartter syndrome type 3: an unusual cause of nephrolithiasis. *Nephrol Dial Transplant* 17: 521-523, 2002.

64. **Com G, and Clancy JP**. Adenosine receptors, cystic fibrosis, and airway hydration. *Handb Exp Pharmacol* 363-381, 2009.
65. **Control CfD**. Asthma in the US [July, 2016].
66. **Coppens JT, Van Winkle LS, Pinkerton K, and Plopper CG**. Distribution of Clara cell secretory protein expression in the tracheobronchial airways of rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol* 292: L1155-1162, 2007.
67. **Corthay A**. How do regulatory T cells work? *Scand J Immunol* 70: 326-336, 2009.
68. **Cowley EA, and Linsdell P**. Characterization of basolateral K+ channels underlying anion secretion in the human airway cell line Calu-3. *J Physiol* 538: 747-757, 2002.
69. **Coyne CB, Gambling TM, Boucher RC, Carson JL, and Johnson LG**. Role of claudin interactions in airway tight junctional permeability. *Am J Physiol Lung Cell Mol Physiol* 285: L1166-1178, 2003.
70. **Crosby LM, and Waters CM**. Epithelial repair mechanisms in the lung. *Am J Physiol Lung Cell Mol Physiol* 298: L715-731, 2010.
71. **Crump RG, Askew GR, Wert SE, Lingrel JB, and Joiner CH**. In situ localization of sodium-potassium ATPase mRNA in developing mouse lung epithelium. *Am J Physiol* 269: L299-308, 1995.
72. **Curiel TJ**. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 117: 1167-1174, 2007.
73. **Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, Krzysiek R, Knutson KL, Daniel B, Zimmermann MC, David O, Burow M, Gordon A, Dhurandhar N, Myers L, Berggren R, Hemminki A, Alvarez RD, Emilie D, Curiel DT, Chen L, and Zou W**. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 9: 562-567, 2003.
74. **D'Amato G, Vitale C, De Martino A, Viegi G, Lanza M, Molino A, Sanduzzi A, Vatrella A, Annesi-Maesano I, and D'Amato M**. Effects on asthma and respiratory allergy of Climate change and air pollution. *Multidiscip Respir Med* 10: 39, 2015.
75. **Danahay H, Atherton H, Jones G, Bridges RJ, and Poll CT**. Interleukin-13 induces a hypersecretory ion transport phenotype in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 282: L226-236, 2002.
76. **Daugherty BL, Mateescu M, Patel AS, Wade K, Kimura S, Gonzales LW, Guttentag S, Ballard PL, and Koval M**. Developmental regulation of claudin localization by fetal alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 287: L1266-1273, 2004.
77. **Davies DE**. Epithelial barrier function and immunity in asthma. *Ann Am Thorac Soc* 11 Suppl 5: S244-251, 2014.
78. **Davis AS, Chertow DS, Moyer JE, Suzich J, Sandouk A, Dorward DW, Logun C, Shelhamer JH, and Taubenberger JK**. Validation of normal human bronchial epithelial cells as a model for influenza A infections in human distal trachea. *J Histochem Cytochem* 63: 312-328, 2015.
79. **de Kluijver J, Schrumpf JA, Evertse CE, Sont JK, Roughley PJ, Rabe KF, Hiemstra PS, Mauad T, and Sterk PJ**. Bronchial matrix and inflammation respond to

inhaled steroids despite ongoing allergen exposure in asthma. *Clin Exp Allergy* 35: 1361-1369, 2005.
80. **Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraibi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, and Staub O**. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. *EMBO J* 20: 7052-7059, 2001.
81. **Devor DC, Bridges RJ, and Pilewski JM**. Pharmacological modulation of ion transport across wild-type and DeltaF508 CFTR-expressing human bronchial epithelia. *Am J Physiol Cell Physiol* 279: C461-479, 2000.
82. **Diakov A, and Korbmacher C**. A novel pathway of epithelial sodium channel activation involves a serum- and glucocorticoid-inducible kinase consensus motif in the C terminus of the channel's alpha-subunit. *J Biol Chem* 279: 38134-38142, 2004.
83. **Diamond JM**. Twenty-first Bowditch lecture. The epithelial junction: bridge, gate, and fence. *Physiologist* 20: 10-18, 1977.
84. **Dickman KG, Hempson SJ, Anderson J, Lippe S, Zhao L, Burakoff R, and Shaw RD**. Rotavirus alters paracellular permeability and energy metabolism in Caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* 279: G757-766, 2000.
85. **Dodrill MW, and Fedan JS**. Lipopolysaccharide hyperpolarizes guinea pig airway epithelium by increasing the activities of the epithelial Na(+) channel and the Na(+)-K(+) pump. *Am J Physiol Lung Cell Mol Physiol* 299: L550-558, 2010.
86. **Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Luders HO, Shi J, Cui J, Richerson GB, and Wang QK**. Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet* 37: 733-738, 2005.
87. **Duncan PJ, and Shipston MJ**. BK Channels and the Control of the Pituitary. *Int Rev Neurobiol* 128: 343-368, 2016.
88. **Ehre C, Ridley C, and Thornton DJ**. Cystic fibrosis: an inherited disease affecting mucin-producing organs. *Int J Biochem Cell Biol* 52: 136-145, 2014.
89. **Ellison RT, 3rd, and Giehl TJ**. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest* 88: 1080-1091, 1991.
90. **Emmer BT, Maric D, and Engman DM**. Molecular mechanisms of protein and lipid targeting to ciliary membranes. *J Cell Sci* 123: 529-536, 2010.
91. **Enuka Y, Hanukoglu I, Edelheit O, Vaknine H, and Hanukoglu A**. Epithelial sodium channels (ENaC) are uniformly distributed on motile cilia in the oviduct and the respiratory airways. *Histochem Cell Biol* 137: 339-353, 2012.
92. **Ergonul Z, Frindt G, and Palmer LG**. Regulation of maturation and processing of ENaC subunits in the rat kidney. *Am J Physiol Renal Physiol* 291: F683-693, 2006.
93. **Erxleben C, Everhart AL, Romeo C, Florance H, Bauer MB, Alcorta DA, Rossie S, Shipston MJ, and Armstrong DL**. Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation. *J Biol Chem* 277: 27045-27052, 2002.
94. **Esther CR, Jr., Alexis NE, and Picher M**. Regulation of airway nucleotides in chronic lung diseases. *Subcell Biochem* 55: 75-93, 2011.

95. **Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatziioannou T, McKeating JA, Bieniasz PD, and Rice CM**. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 446: 801-805, 2007.
96. **Factor P**. Role and regulation of lung Na,K-ATPase. *Cell Mol Biol (Noisy-le-grand)* 47: 347-361, 2001.
97. **Fahy JV, and Dickey BF**. Airway mucus function and dysfunction. *N Engl J Med* 363: 2233-2247, 2010.
98. **Falk MM**. Adherens junctions remain dynamic. *BMC Biol* 8: 34, 2010.
99. **Fan Chung K**. Phosphodiesterase inhibitors in airways disease. *Eur J Pharmacol* 533: 110-117, 2006.
100. **Fedorov IA, Wilson SJ, Davies DE, and Holgate ST**. Epithelial stress and structural remodelling in childhood asthma. *Thorax* 60: 389-394, 2005.
101. **Fischer A, Gluth M, Weege F, Pape UF, Wiedenmann B, Baumgart DC, and Theuring F**. Glucocorticoids regulate barrier function and claudin expression in intestinal epithelial cells via MKP-1. *Am J Physiol Gastrointest Liver Physiol* 306: G218-228, 2014.
102. **Flynn AN, Itani OA, Moninger TO, and Welsh MJ**. Acute regulation of tight junction ion selectivity in human airway epithelia. *Proc Natl Acad Sci U S A* 106: 3591-3596, 2009.
103. **Foster WM, Langenback EG, and Bergofsky EH**. Disassociation in the mucociliary function of central and peripheral airways of asymptomatic smokers. *Am Rev Respir Dis* 132: 633-639, 1985.
104. **Foundation CF**. About Cystic Fibrosis https://www.cff.org/What-is-CF/About-Cystic-Fibrosis/.
105. **Frizzell RA, and Hanrahan JW**. Physiology of epithelial chloride and fluid secretion. *Cold Spring Harb Perspect Med* 2: a009563, 2012.
106. **Frizzell RA, Welsh MJ, and Smith PL**. Hormonal control of chloride secretion by canine tracheal epithelium: an electrophysiologic analysis. *Ann N Y Acad Sci* 372: 558-570, 1981.
107. **Fulcher ML, Gabriel S, Burns KA, Yankaskas JR, and Randell SH**. Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* 107: 183-206, 2005.
108. **Furuse M, Fujita K, Hiiragi T, Fujimoto K, and Tsukita S**. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 141: 1539-1550, 1998.
109. **Galietta LJ, Folli C, Caci E, Pedemonte N, Taddei A, Ravazzolo R, and Zegarra-Moran O**. Effect of inflammatory stimuli on airway ion transport. *Proc Am Thorac Soc* 1: 62-65, 2004.
110. **Galietta LJ, Folli C, Marchetti C, Romano L, Carpani D, Conese M, and Zegarra-Moran O**. Modification of transepithelial ion transport in human cultured bronchial epithelial cells by interferon-gamma. *Am J Physiol Lung Cell Mol Physiol* 278: L1186-1194, 2000.

111. **Galietta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, and Zegarra-Moran O**. IL-4 is a potent modulator of ion transport in the human bronchial epithelium in vitro. *J Immunol* 168: 839-845, 2002.
112. **Gao F, Duan X, Lu X, Liu Y, Zheng L, Ding Z, and Li J**. Novel binding between pre-membrane protein and claudin-1 is required for efficient dengue virus entry. *Biochem Biophys Res Commun* 391: 952-957, 2010.
113. **Garcia GJ, Boucher RC, and Elston TC**. Biophysical model of ion transport across human respiratory epithelia allows quantification of ion permeabilities. *Biophys J* 104: 716-726, 2013.
114. **Gauvreau GM, O'Byrne PM, Boulet LP, Wang Y, Cockcroft D, Bigler J, FitzGerald JM, Boedigheimer M, Davis BE, Dias C, Gorski KS, Smith L, Bautista E, Comeau MR, Leigh R, and Parnes JR**. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N Engl J Med* 370: 2102-2110, 2014.
115. **Ghio AJ, Dailey LA, Soukup JM, Stonehuerner J, Richards JH, and Devlin RB**. Growth of human bronchial epithelial cells at an air-liquid interface alters the response to particle exposure. *Part Fibre Toxicol* 10: 25, 2013.
116. **Goleva E, Hauk PJ, Boguniewicz J, Martin RJ, and Leung DY**. Airway remodeling and lack of bronchodilator response in steroid-resistant asthma. *J Allergy Clin Immunol* 120: 1065-1072, 2007.
117. **Gorodeski GI, Jin W, and Hopfer U**. Extracellular Ca2+ directly regulates tight junctional permeability in the human cervical cell line CaSki. *Am J Physiol* 272: C511-524, 1997.
118. **Gras D, Chanez P, Vachier I, Petit A, and Bourdin A**. Bronchial epithelium as a target for innovative treatments in asthma. *Pharmacol Ther* 140: 290-305, 2013.
119. **Gray TE, Guzman K, Davis CW, Abdullah LH, and Nettesheim P**. Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* 14: 104-112, 1996.
120. **Griesenbach U, Soussi S, Larsen MB, Casamayor I, Dewar A, Regamey N, Bush A, Shah PL, Davies JC, and Alton EW**. Quantification of periciliary fluid height in human airway biopsies is feasible, but not suitable as a biomarker. *Am J Respir Cell Mol Biol* 44: 309-315, 2011.
121. **Grigoryan T, Wend P, Klaus A, and Birchmeier W**. Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev* 22: 2308-2341, 2008.
122. **Gunzel D, Stuiver M, Kausalya PJ, Haisch L, Krug SM, Rosenthal R, Meij IC, Hunziker W, Fromm M, and Muller D**. Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function. *J Cell Sci* 122: 1507-1517, 2009.
123. **Gunzel D, and Yu AS**. Claudins and the modulation of tight junction permeability. *Physiol Rev* 93: 525-569, 2013.
124. **Gunzel D, and Yu AS**. Function and regulation of claudins in the thick ascending limb of Henle. *Pflugers Arch* 458: 77-88, 2009.

125. **Guseh JS, Bores SA, Stanger BZ, Zhou Q, Anderson WJ, Melton DA, and Rajagopal J**. Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. *Development* 136: 1751-1759, 2009.
126. **Hackett NR, Shaykhiev R, Walters MS, Wang R, Zwick RK, Ferris B, Witover B, Salit J, and Crystal RG**. The human airway epithelial basal cell transcriptome. *PLoS One* 6: e18378, 2011.
127. **Hadcock JR, and Malbon CC**. Regulation of beta-adrenergic receptors by "permissive" hormones: glucocorticoids increase steady-state levels of receptor mRNA. *Proc Natl Acad Sci U S A* 85: 8415-8419, 1988.
128. **Hajj R, Baranek T, Le Naour R, Lesimple P, Puchelle E, and Coraux C**. Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties. *Stem Cells* 25: 139-148, 2007.
129. **Hamazaki Y, Itoh M, Sasaki H, Furuse M, and Tsukita S**. Multi-PDZ domain protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. *J Biol Chem* 277: 455-461, 2002.
130. **Hamid Q**. Effects of steroids on inflammation and cytokine gene expression in airway inflammation. *J Allergy Clin Immunol* 112: 636-638, 2003.
131. **Hamilton KL, and Eaton DC**. Single-channel recordings from two types of amiloride-sensitive epithelial Na+ channels. *Membr Biochem* 6: 149-171, 1986.
132. **Hampson G, Konrad MA, and Scoble J**. Familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHNC): compound heterozygous mutation in the claudin 16 (CLDN16) gene. *BMC Nephrol* 9: 12, 2008.
133. **Hartsock A, and Nelson WJ**. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta* 1778: 660-669, 2008.
134. **Hatou S, Yamada M, Mochizuki H, Shiraishi A, Joko T, and Nishida T**. The effects of dexamethasone on the Na,K-ATPase activity and pump function of corneal endothelial cells. *Curr Eye Res* 34: 347-354, 2009.
135. **Hayashi T, Kawakami M, Sasaki S, Katsumata T, Mori H, Yoshida H, and Nakahari T**. ATP regulation of ciliary beat frequency in rat tracheal and distal airway epithelium. *Exp Physiol* 90: 535-544, 2005.
136. **Hede SE, Amstrup J, Klaerke DA, and Novak I**. P2Y2 and P2Y4 receptors regulate pancreatic Ca(2+)-activated K+ channels differently. *Pflugers Arch* 450: 429-436, 2005.
137. **Heijink IH, Marcel Kies P, van Oosterhout AJ, Postma DS, Kauffman HF, and Vellenga E**. Der p, IL-4, and TGF-beta cooperatively induce EGFR-dependent TARC expression in airway epithelium. *Am J Respir Cell Mol Biol* 36: 351-359, 2007.
138. **Heiler S, Mu W, Zoller M, and Thuma F**. The importance of claudin-7 palmitoylation on membrane subdomain localization and metastasis-promoting activities. *Cell Commun Signal* 13: 29, 2015.
139. **Herbort CP, Zografos L, Zwingli M, and Schoeneich M**. Topical retinoic acid in dysplastic and metaplastic keratinization of corneoconjunctival epithelium. *Graefes Arch Clin Exp Ophthalmol* 226: 22-26, 1988.
140. **Higgins CF**. ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 8: 67-113, 1992.

141. **Hirsh AJ, Sabater JR, Zamurs A, Smith RT, Paradiso AM, Hopkins S, Abraham WM, and Boucher RC**. Evaluation of second generation amiloride analogs as therapy for cystic fibrosis lung disease. *J Pharmacol Exp Ther* 311: 929-938, 2004.
142. **Holgate ST**. Mechanisms of asthma and implications for its prevention and treatment: a personal journey. *Allergy Asthma Immunol Res* 5: 343-347, 2013.
143. **Holgate ST**. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 242: 205-219, 2011.
144. **Holgate ST, Roberts G, Arshad HS, Howarth PH, and Davies DE**. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc Am Thorac Soc* 6: 655-659, 2009.
145. **Hollenhorst MI, Richter K, and Fronius M**. Ion transport by pulmonary epithelia. *J Biomed Biotechnol* 2011: 174306, 2011.
146. **Hoorn EJ, Nelson JH, McCormick JA, and Ellison DH**. The WNK kinase network regulating sodium, potassium, and blood pressure. *J Am Soc Nephrol* 22: 605-614, 2011.
147. **Hou J, Paul DL, and Goodenough DA**. Paracellin-1 and the modulation of ion selectivity of tight junctions. *J Cell Sci* 118: 5109-5118, 2005.
148. **Hou J, Renigunta A, Konrad M, Gomes AS, Schneeberger EE, Paul DL, Waldegger S, and Goodenough DA**. Claudin-16 and claudin-19 interact and form a cation-selective tight junction complex. *J Clin Invest* 118: 619-628, 2008.
149. **Hu Q, and Nelson WJ**. Ciliary diffusion barrier: the gatekeeper for the primary cilium compartment. *Cytoskeleton (Hoboken)* 68: 313-324, 2011.
150. **Huang F, Zhang H, Wu M, Yang H, Kudo M, Peters CJ, Woodruff PG, Solberg OD, Donne ML, Huang X, Sheppard D, Fahy JV, Wolters PJ, Hogan BL, Finkbeiner WE, Li M, Jan YN, Jan LY, and Rock JR**. Calcium-activated chloride channel TMEM16A modulates mucin secretion and airway smooth muscle contraction. *Proc Natl Acad Sci U S A* 109: 16354-16359, 2012.
151. **Hughey RP, Bruns JB, Kinlough CL, Harkleroad KL, Tong Q, Carattino MD, Johnson JP, Stockand JD, and Kleyman TR**. Epithelial sodium channels are activated by furin-dependent proteolysis. *J Biol Chem* 279: 18111-18114, 2004.
152. **Hughey RP, Mueller GM, Bruns JB, Kinlough CL, Poland PA, Harkleroad KL, Carattino MD, and Kleyman TR**. Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. *J Biol Chem* 278: 37073-37082, 2003.
153. **Hunt JF, Wang C, and Ford RC**. Cystic fibrosis transmembrane conductance regulator (ABCC7) structure. *Cold Spring Harb Perspect Med* 3: a009514, 2013.
154. **Huynh TP, Barwe SP, Lee SJ, McSpadden R, Franco OE, Hayward SW, Damoiseaux R, Grubbs SS, Petrelli NJ, and Rajasekaran AK**. Glucocorticoids suppress renal cell carcinoma progression by enhancing Na,K-ATPase beta-1 subunit expression. *PLoS One* 10: e0122442, 2015.
155. **Ibanez-Tallon I, Heintz N, and Omran H**. To beat or not to beat: roles of cilia in development and disease. *Hum Mol Genet* 12 Spec No 1: R27-35, 2003.

156. **Ikenouchi J, Suzuki M, Umeda K, Ikeda K, Taguchi R, Kobayashi T, Sato SB, Kobayashi T, Stolz DB, and Umeda M**. Lipid polarity is maintained in absence of tight junctions. *J Biol Chem* 287: 9525-9533, 2012.
157. **Imasato A, Desbois-Mouthon C, Han J, Kai H, Cato AC, Akira S, and Li JD**. Inhibition of p38 MAPK by glucocorticoids via induction of MAPK phosphatase-1 enhances nontypeable Haemophilus influenzae-induced expression of toll-like receptor 2. *J Biol Chem* 277: 47444-47450, 2002.
158. **Ingram JL, and Kraft M**. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* 130: 829-842; quiz 843-824, 2012.
159. **Ismail NA, Baines DL, and Wilson SM**. The phosphorylation of endogenous Nedd4-2 In Na(+)-absorbing human airway epithelial cells. *Eur J Pharmacol* 732: 32-42, 2014.
160. **Itani OA, Auerbach SD, Husted RF, Volk KA, Ageloff S, Knepper MA, Stokes JB, and Thomas CP**. Glucocorticoid-stimulated lung epithelial Na(+) transport is associated with regulated ENaC and sgk1 expression. *Am J Physiol Lung Cell Mol Physiol* 282: L631-641, 2002.
161. **Ito K, Barnes PJ, and Adcock IM**. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol* 20: 6891-6903, 2000.
162. **Iwamoto LM, Fujiwara N, Nakamura KT, and Wada RK**. Na-K-2Cl cotransporter inhibition impairs human lung cellular proliferation. *Am J Physiol Lung Cell Mol Physiol* 287: L510-514, 2004.
163. **Jacobson KA, Costanzi S, Joshi BV, Besada P, Shin DH, Ko H, Ivanov AA, and Mamedova L**. Agonists and antagonists for P2 receptors. *Novartis Found Symp* 276: 58-68; discussion 68-72, 107-112, 275-181, 2006.
164. **Jamora C, and Fuchs E**. Intercellular adhesion, signalling and the cytoskeleton. *Nat Cell Biol* 4: E101-108, 2002.
165. **Jansen HM, Sachs AP, and van Alphen L**. Predisposing conditions to bacterial infections in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 151: 2073-2080, 1995.
166. **Jasti J, Furukawa H, Gonzales EB, and Gouaux E**. Structure of acid-sensing ion channel 1 at 1.9 A resolution and low pH. *Nature* 449: 316-323, 2007.
167. **Judd E, and Calhoun DA**. Management of Resistant Hypertension: Do Not Give Up on Medication. *Nephrol Self Assess Program* 13: 57-63, 2014.
168. **Kaartinen L, Nettesheim P, Adler KB, and Randell SH**. Rat tracheal epithelial cell differentiation in vitro. *In Vitro Cell Dev Biol Anim* 29A: 481-492, 1993.
169. **Kabra R, Knight KK, Zhou R, and Snyder PM**. Nedd4-2 induces endocytosis and degradation of proteolytically cleaved epithelial Na+ channels. *J Biol Chem* 283: 6033-6039, 2008.
170. **Kagoshima M, Ito K, Cosio B, and Adcock IM**. Glucocorticoid suppression of nuclear factor-kappa B: a role for histone modifications. *Biochem Soc Trans* 31: 60-65, 2003.

171. **Kamynina E, and Staub O**. Concerted action of ENaC, Nedd4-2, and Sgk1 in transepithelial Na(+) transport. *Am J Physiol Renal Physiol* 283: F377-387, 2002.
172. **Kao CY, Huang F, Chen Y, Thai P, Wachi S, Kim C, Tam L, and Wu R**. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol* 175: 6676-6685, 2005.
173. **Kashlan OB, Sheng S, and Kleyman TR**. On the interaction between amiloride and its putative alpha-subunit epithelial Na+ channel binding site. *J Biol Chem* 280: 26206-26215, 2005.
174. **Kato A, and Schleimer RP**. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol* 19: 711-720, 2007.
175. **Katzur AC, Koshimizu T, Tomic M, Schultze-Mosgau A, Ortmann O, and Stojilkovic SS**. Expression and responsiveness of P2Y2 receptors in human endometrial cancer cell lines. *J Clin Endocrinol Metab* 84: 4085-4091, 1999.
176. **Kielgast F, Schmidt H, Braubach P, Winkelmann VE, Thompson KE, Frick M, Dietl P, and Wittekindt OH**. Glucocorticoids Regulate Tight Junction Permeability of Lung Epithelia by Modulating Claudin 8. *Am J Respir Cell Mol Biol* 54: 707-717, 2016.
177. **Kim BG, Kim JY, Kim M, Kim CH, Choi JY, and Kim SH**. Gene regulation by glucocorticoid in ENaC-mediated Na(+) transport by middle ear epithelial cells. *Laryngoscope* 124: E27-33, 2014.
178. **Kim EY, Kim J, Morton JD, Swanson S, Patel A, Castro M, and Holtzman MJ**. Glucocorticoids suppress goblet cell metaplasia in natural and experimental asthma. *Journal of Allergy and Clinical Immunology* 113: S116.
179. **Kim J, Myers AC, Chen L, Pardoll DM, Truong-Tran QA, Lane AP, McDyer JF, Fortuno L, and Schleimer RP**. Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol* 33: 280-289, 2005.
180. **Kim JK, Kim SS, Rha KW, Kim CH, Cho JH, Lee CH, Lee JG, and Yoon JH**. Expression and localization of surfactant proteins in human nasal epithelium. *Am J Physiol Lung Cell Mol Physiol* 292: L879-884, 2007.
181. **Kim SA, Tai CY, Mok LP, Mosser EA, and Schuman EM**. Calcium-dependent dynamics of cadherin interactions at cell-cell junctions. *Proc Natl Acad Sci U S A* 108: 9857-9862, 2011.
182. **Kim V, Kelemen SE, Abuel-Haija M, Gaughan JP, Sharafkaneh A, Evans CM, Dickey BF, Solomides CC, Rogers TJ, and Criner GJ**. Small airway mucous metaplasia and inflammation in chronic obstructive pulmonary disease. *COPD* 5: 329-338, 2008.
183. **Kis A, Krick S, Baumlin N, and Salathe M**. Airway Hydration, Apical K(+) Secretion, and the Large-Conductance, Ca(2+)-activated and Voltage-dependent Potassium (BK) Channel. *Ann Am Thorac Soc* 13 Suppl 2: S163-168, 2016.
184. **Knight DA, and Holgate ST**. The airway epithelium: structural and functional properties in health and disease. *Respirology* 8: 432-446, 2003.

185. **Knight KK, Olson DR, Zhou R, and Snyder PM**. Liddle's syndrome mutations increase Na+ transport through dual effects on epithelial Na+ channel surface expression and proteolytic cleavage. *Proc Natl Acad Sci U S A* 103: 2805-2808, 2006.
186. **Kornerup KN, Page CP, and Moffatt JD**. Pharmacological characterisation of the adenosine receptor mediating increased ion transport in the mouse isolated trachea and the effect of allergen challenge. *Br J Pharmacol* 144: 1011-1016, 2005.
187. **Koval M**. Claudin heterogeneity and control of lung tight junctions. *Annu Rev Physiol* 75: 551-567, 2013.
188. **Kreda SM, Gynn MC, Fenstermacher DA, Boucher RC, and Gabriel SE**. Expression and localization of epithelial aquaporins in the adult human lung. *Am J Respir Cell Mol Biol* 24: 224-234, 2001.
189. **Kreda SM, Okada SF, van Heusden CA, O'Neal W, Gabriel S, Abdullah L, Davis CW, Boucher RC, and Lazarowski ER**. Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol* 584: 245-259, 2007.
190. **Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnober EK, Mee C, Soulier E, Royer C, Lambotin M, Grunert F, Dao Thi VL, Dreux M, Cosset FL, McKeating JA, Schuster C, and Baumert TF**. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* 51: 1144-1157, 2010.
191. **Kuhn S, Koch M, Nubel T, Ladwein M, Antolovic D, Klingbeil P, Hildebrand D, Moldenhauer G, Langbein L, Franke WW, Weitz J, and Zoller M**. A complex of EpCAM, claudin-7, CD44 variant isoforms, and tetraspanins promotes colorectal cancer progression. *Mol Cancer Res* 5: 553-567, 2007.
192. **Kunzelmann K, and Mall M**. Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. *Am J Respir Med* 2: 299-309, 2003.
193. **Kunzelmann K, Schreiber R, and Boucherot A**. Mechanisms of the inhibition of epithelial Na(+) channels by CFTR and purinergic stimulation. *Kidney Int* 60: 455-461, 2001.
194. **Kuo T, McQueen A, Chen TC, and Wang JC**. Regulation of Glucose Homeostasis by Glucocorticoids. *Adv Exp Med Biol* 872: 99-126, 2015.
195. **Kyle BD, and Braun AP**. The regulation of BK channel activity by pre- and post-translational modifications. *Front Physiol* 5: 316, 2014.
196. **Kyle BD, Hurst S, Swayze RD, Sheng J, and Braun AP**. Specific phosphorylation sites underlie the stimulation of a large conductance, Ca(2+)-activated K(+) channel by cGMP-dependent protein kinase. *FASEB J* 27: 2027-2038, 2013.
197. **Lai GJ, and McCobb DP**. Opposing actions of adrenal androgens and glucocorticoids on alternative splicing of Slo potassium channels in bovine chromaffin cells. *Proc Natl Acad Sci U S A* 99: 7722-7727, 2002.
198. **Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, and Laitinen LA**. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 156: 951-958, 1997.

199. **Laitinen LA, Heino M, Laitinen A, Kava T, and Haahtela T**. Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* 131: 599-606, 1985.
200. **Lancillotti F, Darwiche N, Celli G, and De Luca LM**. Retinoid status and the control of keratin expression and adhesion during the histogenesis of squamous metaplasia of tracheal epithelium. *Cancer Res* 52: 6144-6152, 1992.
201. **Larsen EH**. Hans H. Ussing--scientific work: contemporary significance and perspectives. *Biochim Biophys Acta* 1566: 2-15, 2002.
202. **Lasa M, Abraham SM, Boucheron C, Saklatvala J, and Clark AR**. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Mol Cell Biol* 22: 7802-7811, 2002.
203. **Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, and Boucher RC**. Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J Biol Chem* 279: 36855-36864, 2004.
204. **Lazrak A, Jurkuvenaite A, Chen L, Keeling KM, Collawn JF, Bedwell DM, and Matalon S**. Enhancement of alveolar epithelial sodium channel activity with decreased cystic fibrosis transmembrane conductance regulator expression in mouse lung. *Am J Physiol Lung Cell Mol Physiol* 301: L557-567, 2011.
205. **Lazrak A, Samanta A, Venetsanou K, Barbry P, and Matalon S**. Modification of biophysical properties of lung epithelial Na(+) channels by dexamethasone. *Am J Physiol Cell Physiol* 279: C762-770, 2000.
206. **Lebowitz J, Edinger RS, An B, Perry CJ, Onate S, Kleyman TR, and Johnson JP**. Ikappab kinase-beta (ikkbeta) modulation of epithelial sodium channel activity. *J Biol Chem* 279: 41985-41990, 2004.
207. **Lee US, and Cui J**. BK channel activation: structural and functional insights. *Trends Neurosci* 33: 415-423, 2010.
208. **Li H, Sheppard DN, and Hug MJ**. Transepithelial electrical measurements with the Ussing chamber. *J Cyst Fibros* 3 Suppl 2: 123-126, 2004.
209. **Lin H, Li H, Cho HJ, Bian S, Roh HJ, Lee MK, Kim JS, Chung SJ, Shim CK, and Kim DD**. Air-liquid interface (ALI) culture of human bronchial epithelial cell monolayers as an in vitro model for airway drug transport studies. *J Pharm Sci* 96: 341-350, 2007.
210. **Linsdell P, Tabcharani JA, and Hanrahan JW**. Multi-Ion mechanism for ion permeation and block in the cystic fibrosis transmembrane conductance regulator chloride channel. *J Gen Physiol* 110: 365-377, 1997.
211. **Linsdell P, Tabcharani JA, Rommens JM, Hou YX, Chang XB, Tsui LC, Riordan JR, and Hanrahan JW**. Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions. *J Gen Physiol* 110: 355-364, 1997.
212. **Livraghi A, and Randell SH**. Cystic fibrosis and other respiratory diseases of impaired mucus clearance. *Toxicol Pathol* 35: 116-129, 2007.

213. **Lorenz S, Heils A, Kasper JM, and Sander T**. Allelic association of a truncation mutation of the KCNMB3 gene with idiopathic generalized epilepsy. *Am J Med Genet B Neuropsychiatr Genet* 144B: 10-13, 2007.
214. **Lu M, MacGregor GG, Wang W, and Giebisch G**. Extracellular ATP inhibits the small-conductance K channel on the apical membrane of the cortical collecting duct from mouse kidney. *J Gen Physiol* 116: 299-310, 2000.
215. **Ma T, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ, and Verkman AS**. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 110: 1651-1658, 2002.
216. **Madara JL**. Sodium-glucose cotransport and epithelial permeability. *Gastroenterology* 107: 319-320, 1994.
217. **Mall M, Bleich M, Greger R, Schreiber R, and Kunzelmann K**. The amiloride-inhibitable Na+ conductance is reduced by the cystic fibrosis transmembrane conductance regulator in normal but not in cystic fibrosis airways. *J Clin Invest* 102: 15-21, 1998.
218. **Mall M, Gonska T, Thomas J, Schreiber R, Seydewitz HH, Kuehr J, Brandis M, and Kunzelmann K**. Modulation of Ca2+-activated Cl- secretion by basolateral K+ channels in human normal and cystic fibrosis airway epithelia. *Pediatr Res* 53: 608-618, 2003.
219. **Mall M, Wissner A, Schreiber R, Kuehr J, Seydewitz HH, Brandis M, Greger R, and Kunzelmann K**. Role of K(V)LQT1 in cyclic adenosine monophosphate-mediated Cl(-) secretion in human airway epithelia. *Am J Respir Cell Mol Biol* 23: 283-289, 2000.
220. **Mall MA**. Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *J Aerosol Med Pulm Drug Deliv* 21: 13-24, 2008.
221. **Mandel LJ, Bacallao R, and Zampighi G**. Uncoupling of the molecular 'fence' and paracellular 'gate' functions in epithelial tight junctions. *Nature* 361: 552-555, 1993.
222. **Mano K, Akbarzadeh A, and Townley RG**. Effect of hydrocortisone on beta-adrenergic receptors in lung membranes. *Life Sci* 25: 1925-1930, 1979.
223. **Manzanares D, Gonzalez C, Ivonnet P, Chen RS, Valencia-Gattas M, Conner GE, Larsson HP, and Salathe M**. Functional apical large conductance, Ca2+-activated, and voltage-dependent K+ channels are required for maintenance of airway surface liquid volume. *J Biol Chem* 286: 19830-19839, 2011.
224. **Manzanares D, Krick S, Baumlin N, Dennis JS, Tyrrell J, Tarran R, and Salathe M**. Airway Surface Dehydration by Transforming Growth Factor beta (TGF-beta) in Cystic Fibrosis Is Due to Decreased Function of a Voltage-dependent Potassium Channel and Can Be Rescued by the Drug Pirfenidone. *J Biol Chem* 290: 25710-25716, 2015.
225. **Manzanares D, Srinivasan M, Salathe ST, Ivonnet P, Baumlin N, Dennis JS, Conner GE, and Salathe M**. IFN-gamma-mediated reduction of large-conductance, Ca2+-activated, voltage-dependent K+ (BK) channel activity in airway epithelial cells

leads to mucociliary dysfunction. *Am J Physiol Lung Cell Mol Physiol* 306: L453-462, 2014.
226. **Marcet B, Horckmans M, Libert F, Hassid S, Boeynaems JM, and Communi D**. Extracellular nucleotides regulate CCL20 release from human primary airway epithelial cells, monocytes and monocyte-derived dendritic cells. *J Cell Physiol* 211: 716-727, 2007.
227. **Marcus DC, Sunose H, Liu J, Shen Z, and Scofield MA**. P2U purinergic receptor inhibits apical IsK/KvLQT1 channel via protein kinase C in vestibular dark cells. *Am J Physiol* 273: C2022-2029, 1997.
228. **Martinez-Palomo A, Meza I, Beaty G, and Cereijido M**. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J Cell Biol* 87: 736-745, 1980.
229. **Matalon S, Bartoszewski R, and Collawn JF**. Role of epithelial sodium channels in the regulation of lung fluid homeostasis. *Am J Physiol Lung Cell Mol Physiol* 309: L1229-1238, 2015.
230. **Mathai JC, Tristram-Nagle S, Nagle JF, and Zeidel ML**. Structural determinants of water permeability through the lipid membrane. *J Gen Physiol* 131: 69-76, 2008.
231. **Mathis C, Poussin C, Weisensee D, Gebel S, Hengstermann A, Sewer A, Belcastro V, Xiang Y, Ansari S, Wagner S, Hoeng J, and Peitsch MC**. Human bronchial epithelial cells exposed in vitro to cigarette smoke at the air-liquid interface resemble bronchial epithelium from human smokers. *Am J Physiol Lung Cell Mol Physiol* 304: L489-503, 2013.
232. **Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, and Boucher RC**. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 95: 1005-1015, 1998.
233. **Matsui H, Randell SH, Peretti SW, Davis CW, and Boucher RC**. Coordinated clearance of periciliary liquid and mucus from airway surfaces. *J Clin Invest* 102: 1125-1131, 1998.
234. **Matter K, and Balda MS**. Functional analysis of tight junctions. *Methods* 30: 228-234, 2003.
235. **McEneaney V, Harvey BJ, and Thomas W**. Aldosterone regulates rapid trafficking of epithelial sodium channel subunits in renal cortical collecting duct cells via protein kinase D activation. *Mol Endocrinol* 22: 881-892, 2008.
236. **McNicholas CM, Yang Y, Giebisch G, and Hebert SC**. Molecular site for nucleotide binding on an ATP-sensitive renal K+ channel (ROMK2). *Am J Physiol* 271: F275-285, 1996.
237. **Mercier-Zuber A, and O'Shaughnessy KM**. Role of SPAK and OSR1 signalling in the regulation of NaCl cotransporters. *Curr Opin Nephrol Hypertens* 20: 534-540, 2011.
238. **Mezey RJ, Cohn MA, Fernandez RJ, Januszkiewicz AJ, and Wanner A**. Mucociliary transport in allergic patients with antigen-induced bronchospasm. *Am Rev Respir Dis* 118: 677-684, 1978.

239. **Million K, Tournier F, Houcine O, Ancian P, Reichert U, and Marano F**. Effects of retinoic acid receptor-selective agonists on human nasal epithelial cell differentiation. *Am J Respir Cell Mol Biol* 25: 744-750, 2001.
240. **Mitchell C, Syed NI, Gurney AM, and Kennedy C**. A Ca(2)(+)-dependent chloride current and Ca(2)(+) influx via Ca(v)1.2 ion channels play major roles in P2Y receptor-mediated pulmonary vasoconstriction. *Br J Pharmacol* 166: 1503-1512, 2012.
241. **Mitchell LA, Overgaard CE, Ward C, Margulies SS, and Koval M**. Differential effects of claudin-3 and claudin-4 on alveolar epithelial barrier function. *Am J Physiol Lung Cell Mol Physiol* 301: L40-49, 2011.
242. **Mo D, Potter BA, Bertrand CA, Hildebrand JD, Bruns JR, and Weisz OA**. Nucleofection disrupts tight junction fence function to alter membrane polarity of renal epithelial cells. *Am J Physiol Renal Physiol* 299: F1178-1184, 2010.
243. **Montes-Vizuet R, Vega-Miranda A, Valencia-Maqueda E, Negrete-Garcia MC, Velasquez JR, and Teran LM**. CC chemokine ligand 1 is released into the airways of atopic asthmatics. *Eur Respir J* 28: 59-67, 2006.
244. **Mou H, Zhao R, Sherwood R, Ahfeldt T, Lapey A, Wain J, Sicilian L, Izvolsky K, Musunuru K, Cowan C, and Rajagopal J**. Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. *Cell Stem Cell* 10: 385-397, 2012.
245. **Mummery JL, Killey J, and Linsdell P**. Expression of the chloride channel CLC-K in human airway epithelial cells. *Can J Physiol Pharmacol* 83: 1123-1128, 2005.
246. **Murdoch JR, and Lloyd CM**. Chronic inflammation and asthma. *Mutat Res* 690: 24-39, 2010.
247. **Nachury MV, Seeley ES, and Jin H**. Trafficking to the ciliary membrane: how to get across the periciliary diffusion barrier? *Annu Rev Cell Dev Biol* 26: 59-87, 2010.
248. **Nagle JF, Mathai JC, Zeidel ML, and Tristram-Nagle S**. Theory of passive permeability through lipid bilayers. *J Gen Physiol* 131: 77-85, 2008.
249. **Nakahari T**. Regulation of ciliary beat frequency in airways: shear stress, ATP action, and its modulation. *Am J Physiol Lung Cell Mol Physiol* 292: L612-613, 2007.
250. **Nakajima M, Kawanami O, Jin E, Ghazizadeh M, Honda M, Asano G, Horiba K, and Ferrans VJ**. Immunohistochemical and ultrastructural studies of basal cells, Clara cells and bronchiolar cuboidal cells in normal human airways. *Pathol Int* 48: 944-953, 1998.
251. **Nakano Y, Kim SH, Kim HM, Sanneman JD, Zhang Y, Smith RJ, Marcus DC, Wangemann P, Nessler RA, and Banfi B**. A claudin-9-based ion permeability barrier is essential for hearing. *PLoS Genet* 5: e1000610, 2009.
252. **Namkung W, Phuan PW, and Verkman AS**. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. *J Biol Chem* 286: 2365-2374, 2011.
253. **Naren AP, Cobb B, Li C, Roy K, Nelson D, Heda GD, Liao J, Kirk KL, Sorscher EJ, Hanrahan J, and Clancy JP**. A macromolecular complex of beta 2 adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. *Proc Natl Acad Sci U S A* 100: 342-346, 2003.

254. **Nava P, Lopez S, Arias CF, Islas S, and Gonzalez-Mariscal L**. The rotavirus surface protein VP8 modulates the gate and fence function of tight junctions in epithelial cells. *J Cell Sci* 117: 5509-5519, 2004.
255. **Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, Arsenault AL, and Kaushic C**. Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog* 6: e1000852, 2010.
256. **Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, and Jetten AM**. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. *Exp Cell Res* 195: 163-170, 1991.
257. **Nicholas RA, Watt WC, Lazarowski ER, Li Q, and Harden K**. Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol Pharmacol* 50: 224-229, 1996.
258. **NIH N**. What is Asthma? [July, 2016].
259. **Nino G, Hu A, Grunstein JS, and Grunstein MM**. Mechanism of glucocorticoid protection of airway smooth muscle from proasthmatic effects of long-acting beta2-adrenoceptor agonist exposure. *J Allergy Clin Immunol* 125: 1020-1027, 2010.
260. **O'Brodovich HM**. Immature epithelial Na+ channel expression is one of the pathogenetic mechanisms leading to human neonatal respiratory distress syndrome. *Proc Assoc Am Physicians* 108: 345-355, 1996.
261. **Ochs M, Nyengaard JR, Jung A, Knudsen L, Voigt M, Wahlers T, Richter J, and Gundersen HJ**. The number of alveoli in the human lung. *Am J Respir Crit Care Med* 169: 120-124, 2004.
262. **Okada T, Nakamura T, Watanabe T, Onoda N, Ashida A, Okuyama R, and Ito K**. Coexpression of EpCAM, CD44 variant isoforms and claudin-7 in anaplastic thyroid carcinoma. *PLoS One* 9: e94487, 2014.
263. **Ostedgaard LS, Baldursson O, and Welsh MJ**. Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by its R domain. *J Biol Chem* 276: 7689-7692, 2001.
264. **Ostrowski LE, and Nettesheim P**. Inhibition of ciliated cell differentiation by fluid submersion. *Exp Lung Res* 21: 957-970, 1995.
265. **Overgaard CE, Daugherty BL, Mitchell LA, and Koval M**. Claudins: control of barrier function and regulation in response to oxidant stress. *Antioxid Redox Signal* 15: 1179-1193, 2011.
266. **Overgaard CE, Mitchell LA, and Koval M**. Roles for claudins in alveolar epithelial barrier function. *Ann N Y Acad Sci* 1257: 167-174, 2012.
267. **Parker D, and Prince A**. Innate immunity in the respiratory epithelium. *Am J Respir Cell Mol Biol* 45: 189-201, 2011.
268. **Paz C, Cornejo Maciel F, Gorostizaga A, Castillo AF, Mori Sequeiros Garcia MM, Maloberti PM, Orlando UD, Mele PG, Poderoso C, and Podesta EJ**. Role of Protein Phosphorylation and Tyrosine Phosphatases in the Adrenal Regulation of Steroid Synthesis and Mitochondrial Function. *Front Endocrinol (Lausanne)* 7: 60, 2016.

269. **Pearce N, Pekkanen J, and Beasley R**. How much asthma is really attributable to atopy? *Thorax* 54: 268-272, 1999.
270. **Peitzman ER, Zaidman NA, Maniak PJ, and O'Grady SM**. Agonist binding to beta-adrenergic receptors on human airway epithelial cells inhibits migration and wound repair. *Am J Physiol Cell Physiol* 309: C847-855, 2015.
271. **Peitzman ER, Zaidman NA, Maniak PJ, and O'Grady SM**. Carvedilol binding to beta2-adrenergic receptors inhibits CFTR-dependent anion secretion in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 310: L50-58, 2016.
272. **Petrik D, and Brenner R**. Regulation of STREX exon large conductance, calcium-activated potassium channels by the beta4 accessory subunit. *Neuroscience* 149: 789-803, 2007.
273. **Phadke AP, Akangire G, Park SJ, Lira SA, and Mehrad B**. The role of CC chemokine receptor 6 in host defense in a model of invasive pulmonary aspergillosis. *Am J Respir Crit Care Med* 175: 1165-1172, 2007.
274. **Pichavant M, Taront S, Jeannin P, Breuilh L, Charbonnier AS, Spriet C, Fourneau C, Corvaia N, Heliot L, Brichet A, Tonnel AB, Delneste Y, and Gosset P**. Impact of bronchial epithelium on dendritic cell migration and function: modulation by the bacterial motif KpOmpA. *J Immunol* 177: 5912-5919, 2006.
275. **Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, and Abrignani S**. Binding of hepatitis C virus to CD81. *Science* 282: 938-941, 1998.
276. **Planes C, Randrianarison NH, Charles RP, Frateschi S, Cluzeaud F, Vuagniaux G, Soler P, Clerici C, Rossier BC, and Hummler E**. ENaC-mediated alveolar fluid clearance and lung fluid balance depend on the channel-activating protease 1. *EMBO Mol Med* 2: 26-37, 2010.
277. **Pochynyuk O, Bugaj V, and Stockand JD**. Physiologic regulation of the epithelial sodium channel by phosphatidylinositides. *Curr Opin Nephrol Hypertens* 17: 533-540, 2008.
278. **Pochynyuk O, Bugaj V, Vandewalle A, and Stockand JD**. Purinergic control of apical plasma membrane PI(4,5)P2 levels sets ENaC activity in principal cells. *Am J Physiol Renal Physiol* 294: F38-46, 2008.
279. **Pohl C, Hermanns MI, Uboldi C, Bock M, Fuchs S, Dei-Anang J, Mayer E, Kehe K, Kummer W, and Kirkpatrick CJ**. Barrier functions and paracellular integrity in human cell culture models of the proximal respiratory unit. *Eur J Pharm Biopharm* 72: 339-349, 2009.
280. **Postma DS, and Kerstjens HA**. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158: S187-192, 1998.
281. **Poulsen H, Nissen P, Mouritsen OG, and Khandelia H**. Protein kinase A (PKA) phosphorylation of Na+/K+-ATPase opens intracellular C-terminal water pathway leading to third Na+-binding site in molecular dynamics simulations. *J Biol Chem* 287: 15959-15965, 2012.
282. **Powell DW**. Barrier function of epithelia. *Am J Physiol* 241: G275-288, 1981.

283. **Price MP, Ishihara H, Sheppard DN, and Welsh MJ**. Function of Xenopus cystic fibrosis transmembrane conductance regulator (CFTR) Cl channels and use of human-Xenopus chimeras to investigate the pore properties of CFTR. *J Biol Chem* 271: 25184-25191, 1996.
284. **Proud D, and Leigh R**. Epithelial cells and airway diseases. *Immunol Rev* 242: 186-204, 2011.
285. **Puchelle E, Zahm JM, Tournier JM, and Coraux C**. Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 3: 726-733, 2006.
286. **Qu BH, and Thomas PJ**. Alteration of the cystic fibrosis transmembrane conductance regulator folding pathway. *J Biol Chem* 271: 7261-7264, 1996.
287. **Quesnell RR, Han X, and Schultz BD**. Glucocorticoids stimulate ENaC upregulation in bovine mammary epithelium. *Am J Physiol Cell Physiol* 292: C1739-1745, 2007.
288. **Rackley CR, and Stripp BR**. Building and maintaining the epithelium of the lung. *J Clin Invest* 122: 2724-2730, 2012.
289. **Raju SV, and Wang G**. Suppression of adenosine-activated chloride transport by ethanol in airway epithelia. *PLoS One* 7: e32112, 2012.
290. **Reber LL, Daubeuf F, Plantinga M, De Cauwer L, Gerlo S, Waelput W, Van Calenbergh S, Tavernier J, Haegeman G, Lambrecht BN, Frossard N, and De Bosscher K**. A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma. *J Immunol* 188: 3478-3487, 2012.
291. **Reibman J, Hsu Y, Chen LC, Bleck B, and Gordon T**. Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol* 28: 648-654, 2003.
292. **Rock JR, Gao X, Xue Y, Randell SH, Kong YY, and Hogan BL**. Notch-dependent differentiation of adult airway basal stem cells. *Cell Stem Cell* 8: 639-648, 2011.
293. **Rock JR, and Hogan BL**. Epithelial progenitor cells in lung development, maintenance, repair, and disease. *Annu Rev Cell Dev Biol* 27: 493-512, 2011.
294. **Rock JR, O'Neal WK, Gabriel SE, Randell SH, Harfe BD, Boucher RC, and Grubb BR**. Transmembrane protein 16A (TMEM16A) is a Ca2+-regulated Cl- secretory channel in mouse airways. *J Biol Chem* 284: 14875-14880, 2009.
295. **Rock JR, Onaitis MW, Rawlins EL, Lu Y, Clark CP, Xue Y, Randell SH, and Hogan BL**. Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci U S A* 106: 12771-12775, 2009.
296. **Rock JR, Randell SH, and Hogan BL**. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis Model Mech* 3: 545-556, 2010.
297. **Romagnani S**. T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* 85: 9-18; quiz 18, 21, 2000.
298. **Rose MC, and Voynow JA**. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev* 86: 245-278, 2006.

299. **Rosenthal R, Milatz S, Krug SM, Oelrich B, Schulzke JD, Amasheh S, Gunzel D, and Fromm M**. Claudin-2, a component of the tight junction, forms a paracellular water channel. *J Cell Sci* 123: 1913-1921, 2010.
300. **Ross AJ, Dailey LA, Brighton LE, and Devlin RB**. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. *Am J Respir Cell Mol Biol* 37: 169-185, 2007.
301. **Rossi A, Khirani S, and Cazzola M**. Long-acting beta2-agonists (LABA) in chronic obstructive pulmonary disease: efficacy and safety. *Int J Chron Obstruct Pulmon Dis* 3: 521-529, 2008.
302. **Rossier BC**. Epithelial sodium channel (ENaC) and the control of blood pressure. *Curr Opin Pharmacol* 15: 33-46, 2014.
303. **Rowe SM, and Verkman AS**. Cystic fibrosis transmembrane regulator correctors and potentiators. *Cold Spring Harb Perspect Med* 3: 2013.
304. **Rubenstein RC, Lockwood SR, Lide E, Bauer R, Suaud L, and Grumbach Y**. Regulation of endogenous ENaC functional expression by CFTR and DeltaF508-CFTR in airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 300: L88-L101, 2011.
305. **Rubin BK**. Physiology of airway mucus clearance. *Respir Care* 47: 761-768, 2002.
306. **Ruckes-Nilges C, Weber U, Lindemann H, Munker G, Clauss W, and Weber WM**. Minor role of Cl- secretion in non-cystic fibrosis and cystic fibrosis human nasal epithelium. *Cell Physiol Biochem* 9: 1-10, 1999.
307. **Sachs LA, Finkbeiner WE, and Widdicombe JH**. Effects of media on differentiation of cultured human tracheal epithelium. *In Vitro Cell Dev Biol Anim* 39: 56-62, 2003.
308. **Sachse G, Faulhaber J, Seniuk A, Ehmke H, and Pongs O**. Smooth muscle BK channel activity influences blood pressure independent of vascular tone in mice. *J Physiol* 592: 2563-2574, 2014.
309. **Sagel SD, Sontag MK, and Accurso FJ**. Relationship between antimicrobial proteins and airway inflammation and infection in cystic fibrosis. *Pediatr Pulmonol* 44: 402-409, 2009.
310. **Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, Noda T, and Tsukita S**. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* 11: 4131-4142, 2000.
311. **Salyer SA, Parks J, Barati MT, Lederer ED, Clark BJ, Klein JD, and Khundmiri SJ**. Aldosterone regulates Na(+), K(+) ATPase activity in human renal proximal tubule cells through mineralocorticoid receptor. *Biochim Biophys Acta* 1833: 2143-2152, 2013.
312. **Savalli N, Pantazis A, Yusifov T, Sigg D, and Olcese R**. The contribution of RCK domains to human BK channel allosteric activation. *J Biol Chem* 287: 21741-21750, 2012.
313. **Sayegh R, Auerbach SD, Li X, Loftus RW, Husted RF, Stokes JB, and Thomas CP**. Glucocorticoid induction of epithelial sodium channel expression in lung and renal epithelia occurs via trans-activation of a hormone response element in the 5'-

flanking region of the human epithelial sodium channel alpha subunit gene. *J Biol Chem* 274: 12431-12437, 1999.
314. **Schaller MA, Lundy SK, Huffnagle GB, and Lukacs NW**. CD8+ T cell contributions to allergen induced pulmonary inflammation and airway hyperreactivity. *Eur J Immunol* 35: 2061-2070, 2005.
315. **Schild L**. The epithelial sodium channel and the control of sodium balance. *Biochim Biophys Acta* 1802: 1159-1165, 2010.
316. **Schmid A, Sutto Z, Nlend MC, Horvath G, Schmid N, Buck J, Levin LR, Conner GE, Fregien N, and Salathe M**. Soluble adenylyl cyclase is localized to cilia and contributes to ciliary beat frequency regulation via production of cAMP. *J Gen Physiol* 130: 99-109, 2007.
317. **Schmittgen TD, and Livak KJ**. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101-1108, 2008.
318. **Schneeberger EE, and Lynch RD**. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol* 286: C1213-1228, 2004.
319. **Schreiber R, and Kunzelmann K**. Purinergic P2Y6 receptors induce Ca2+ and CFTR dependent Cl- secretion in mouse trachea. *Cell Physiol Biochem* 16: 99-108, 2005.
320. **Schreiber R, Nitschke R, Greger R, and Kunzelmann K**. The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. *J Biol Chem* 274: 11811-11816, 1999.
321. **Schroder K, Hertzog PJ, Ravasi T, and Hume DA**. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75: 163-189, 2004.
322. **Schuliga M**. NF-kappaB Signaling in Chronic Inflammatory Airway Disease. *Biomolecules* 5: 1266-1283, 2015.
323. **Schulzke JD, Gitter AH, Mankertz J, Spiegel S, Seidler U, Amasheh S, Saitou M, Tsukita S, and Fromm M**. Epithelial transport and barrier function in occludin-deficient mice. *Biochim Biophys Acta* 1669: 34-42, 2005.
324. **Schwiebert EM, Kizer N, Gruenert DC, and Stanton BA**. GTP-binding proteins inhibit cAMP activation of chloride channels in cystic fibrosis airway epithelial cells. *Proc Natl Acad Sci U S A* 89: 10623-10627, 1992.
325. **Sears PR, Yin WN, and Ostrowski LE**. Continuous mucociliary transport by primary human airway epithelial cells in vitro. *Am J Physiol Lung Cell Mol Physiol* 309: L99-108, 2015.
326. **Senatorov VV, Stys PK, and Hu B**. Regulation of Na+,K+-ATPase by persistent sodium accumulation in adult rat thalamic neurones. *J Physiol* 525 Pt 2: 343-353, 2000.
327. **Shah AS, Ben-Shahar Y, Moninger TO, Kline JN, and Welsh MJ**. Motile cilia of human airway epithelia are chemosensory. *Science* 325: 1131-1134, 2009.
328. **Shen KZ, Lagrutta A, Davies NW, Standen NB, Adelman JP, and North RA**. Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: evidence for tetrameric channel formation. *Pflugers Arch* 426: 440-445, 1994.
329. **Sheng S, Carattino MD, Bruns JB, Hughey RP, and Kleyman TR**. Furin cleavage activates the epithelial Na+ channel by relieving Na+ self-inhibition. *Am J Physiol Renal Physiol* 290: F1488-1496, 2006.

330. **Shi H, Asher C, Chigaev A, Yung Y, Reuveny E, Seger R, and Garty H**. Interactions of beta and gamma ENaC with Nedd4 can be facilitated by an ERK-mediated phosphorylation. *J Biol Chem* 277: 13539-13547, 2002.
331. **Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M, Gill JR, Jr., Ulick S, Milora RV, Findling JW, and et al.** Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell* 79: 407-414, 1994.
332. **Shipston MJ, and Tian L**. Posttranscriptional and Posttranslational Regulation of BK Channels. *Int Rev Neurobiol* 128: 91-126, 2016.
333. **Shuto T, Imasato A, Jono H, Sakai A, Xu H, Watanabe T, Rixter DD, Kai H, Andalibi A, Linthicum F, Guan YL, Han J, Cato AC, Lim DJ, Akira S, and Li JD**. Glucocorticoids synergistically enhance nontypeable Haemophilus influenzae-induced Toll-like receptor 2 expression via a negative cross-talk with p38 MAP kinase. *J Biol Chem* 277: 17263-17270, 2002.
334. **Singh AK, Devor DC, Gerlach AC, Gondor M, Pilewski JM, and Bridges RJ**. Stimulation of Cl(-) secretion by chlorzoxazone. *J Pharmacol Exp Ther* 292: 778-787, 2000.
335. **Singh S, Syme CA, Singh AK, Devor DC, and Bridges RJ**. Benzimidazolone activators of chloride secretion: potential therapeutics for cystic fibrosis and chronic obstructive pulmonary disease. *J Pharmacol Exp Ther* 296: 600-611, 2001.
336. **Skold CM**. Remodeling in asthma and COPD--differences and similarities. *Clin Respir J* 4 Suppl 1: 20-27, 2010.
337. **Smolders-de Haas H, Neuvel J, Schmand B, Treffers PE, Koppe JG, and Hoeks J**. Physical development and medical history of children who were treated antenatally with corticosteroids to prevent respiratory distress syndrome: a 10- to 12-year follow-up. *Pediatrics* 86: 65-70, 1990.
338. **Snyder PM, Olson DR, Kabra R, Zhou R, and Steines JC**. cAMP and serum and glucocorticoid-inducible kinase (SGK) regulate the epithelial Na(+) channel through convergent phosphorylation of Nedd4-2. *J Biol Chem* 279: 45753-45758, 2004.
339. **Sondo E, Caci E, and Galietta LJ**. The TMEM16A chloride channel as an alternative therapeutic target in cystic fibrosis. *Int J Biochem Cell Biol* 52: 73-76, 2014.
340. **Soundararajan R, Pearce D, and Ziera T**. The role of the ENaC-regulatory complex in aldosterone-mediated sodium transport. *Mol Cell Endocrinol* 350: 242-247, 2012.
341. **Stannard W, and O'Callaghan C**. Ciliary function and the role of cilia in clearance. *J Aerosol Med* 19: 110-115, 2006.
342. **Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, and Rotin D**. WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. *EMBO J* 15: 2371-2380, 1996.
343. **Stellato C**. Glucocorticoid actions on airway epithelial responses in immunity: functional outcomes and molecular targets. *J Allergy Clin Immunol* 120: 1247-1263; quiz 1264-1245, 2007.
344. **Stellato C**. Post-transcriptional and nongenomic effects of glucocorticoids. *Proc Am Thorac Soc* 1: 255-263, 2004.

345. **Stephanova DI**. Myelin as longitudinal conductor: a multi-layered model of the myelinated human motor nerve fibre. *Biol Cybern* 84: 301-308, 2001.
346. **Stewart CE, Torr EE, Mohd Jamili NH, Bosquillon C, and Sayers I**. Evaluation of differentiated human bronchial epithelial cell culture systems for asthma research. *J Allergy (Cairo)* 2012: 943982, 2012.
347. **Storm JF**. Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. *J Physiol* 385: 733-759, 1987.
348. **Sweet TB, and Cox DH**. Measurements of the BKCa channel's high-affinity Ca2+ binding constants: effects of membrane voltage. *J Gen Physiol* 132: 491-505, 2008.
349. **Szkotak AJ, Murthy M, MacVinish LJ, Duszyk M, and Cuthbert AW**. 4-Chloro-benzo[F]isoquinoline (CBIQ) activates CFTR chloride channels and KCNN4 potassium channels in Calu-3 human airway epithelial cells. *Br J Pharmacol* 142: 531-542, 2004.
350. **Tabcharani JA, Chang XB, Riordan JR, and Hanrahan JW**. The cystic fibrosis transmembrane conductance regulator chloride channel. Iodide block and permeation. *Biophys J* 62: 1-4, 1992.
351. **Tabcharani JA, Rommens JM, Hou YX, Chang XB, Tsui LC, Riordan JR, and Hanrahan JW**. Multi-ion pore behaviour in the CFTR chloride channel. *Nature* 366: 79-82, 1993.
352. **Taddei A, Folli C, Zegarra-Moran O, Fanen P, Verkman AS, and Galietta LJ**. Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. *FEBS Lett* 558: 52-56, 2004.
353. **Tagaya E, and Tamaoki J**. Mechanisms of airway remodeling in asthma. *Allergol Int* 56: 331-340, 2007.
354. **Takakuwa R, Kokai Y, Kojima T, Akatsuka T, Tobioka H, Sawada N, and Mori M**. Uncoupling of gate and fence functions of MDCK cells by the actin-depolymerizing reagent mycalolide B. *Exp Cell Res* 257: 238-244, 2000.
355. **Takao D, and Verhey KJ**. Gated entry into the ciliary compartment. *Cell Mol Life Sci* 73: 119-127, 2016.
356. **Tarran R**. Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc* 1: 42-46, 2004.
357. **Tarran R, Trout L, Donaldson SH, and Boucher RC**. Soluble mediators, not cilia, determine airway surface liquid volume in normal and cystic fibrosis superficial airway epithelia. *J Gen Physiol* 127: 591-604, 2006.
358. **Tashkin DP, Rennard SI, Martin P, Ramachandran S, Martin UJ, Silkoff PE, and Goldman M**. Efficacy and safety of budesonide and formoterol in one pressurized metered-dose inhaler in patients with moderate to very severe chronic obstructive pulmonary disease: results of a 6-month randomized clinical trial. *Drugs* 68: 1975-2000, 2008.
359. **Tawar RG, Colpitts CC, Lupberger J, El-Saghire H, Zeisel MB, and Baumert TF**. Claudins and pathogenesis of viral infection. *Semin Cell Dev Biol* 42: 39-46, 2015.
360. **Terada N, Nomura T, Kim WJ, Otsuka Y, Takahashi R, Kishi H, Yamashita T, Sugawara N, Fukuda S, Ikeda-Ito T, and Konno A**. Expression of C-C chemokine

TARC in human nasal mucosa and its regulation by cytokines. *Clin Exp Allergy* 31: 1923-1931, 2001.
361. **Tessier GJ, Traynor TR, Kannan MS, and O'Grady SM**. Mechanisms of sodium and chloride transport across equine tracheal epithelium. *Am J Physiol* 259: L459-467, 1990.
362. **Tetley TD**. Inflammatory cells and chronic obstructive pulmonary disease. *Curr Drug Targets Inflamm Allergy* 4: 607-618, 2005.
363. **Thavagnanam S, Parker JC, McBrien ME, Skibinski G, Heaney LG, and Shields MD**. Effects of IL-13 on mucociliary differentiation of pediatric asthmatic bronchial epithelial cells. *Pediatr Res* 69: 95-100, 2011.
364. **Thompson AB, Bohling T, Payvandi F, and Rennard SI**. Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. *J Lab Clin Med* 115: 148-158, 1990.
365. **Tian L, Coghill LS, McClafferty H, MacDonald SH, Antoni FA, Ruth P, Knaus HG, and Shipston MJ**. Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc Natl Acad Sci U S A* 101: 11897-11902, 2004.
366. **Tian L, Duncan RR, Hammond MS, Coghill LS, Wen H, Rusinova R, Clark AG, Levitan IB, and Shipston MJ**. Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* 276: 7717-7720, 2001.
367. **Tian X, Liu Z, Niu B, Zhang J, Tan TK, Lee SR, Zhao Y, Harris DC, and Zheng G**. E-cadherin/beta-catenin complex and the epithelial barrier. *J Biomed Biotechnol* 2011: 567305, 2011.
368. **Tilley AE, Walters MS, Shaykhiev R, and Crystal RG**. Cilia dysfunction in lung disease. *Annu Rev Physiol* 77: 379-406, 2015.
369. **Tsukita S, and Furuse M**. Claudin-based barrier in simple and stratified cellular sheets. *Curr Opin Cell Biol* 14: 531-536, 2002.
370. **Turner JR, Rill BK, Carlson SL, Carnes D, Kerner R, Mrsny RJ, and Madara JL**. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. *Am J Physiol* 273: C1378-1385, 1997.
371. **Ueno K, Koga T, Kato K, Golenbock DT, Gendler SJ, Kai H, and Kim KC**. MUC1 mucin is a negative regulator of toll-like receptor signaling. *Am J Respir Cell Mol Biol* 38: 263-268, 2008.
372. **Ueno M**. Mechanisms of the penetration of blood-borne substances into the brain. *Curr Neuropharmacol* 7: 142-149, 2009.
373. **Vagin O, Turdikulova S, and Tokhtaeva E**. Polarized membrane distribution of potassium-dependent ion pumps in epithelial cells: different roles of the N-glycans of their beta subunits. *Cell Biochem Biophys* 47: 376-391, 2007.
374. **Van Itallie CM, and Anderson JM**. The molecular physiology of tight junction pores. *Physiology (Bethesda)* 19: 331-338, 2004.
375. **Van Itallie CM, Fanning AS, Holmes J, and Anderson JM**. Occludin is required for cytokine-induced regulation of tight junction barriers. *J Cell Sci* 123: 2844-2852, 2010.

376. **Van Itallie CM, Rogan S, Yu A, Vidal LS, Holmes J, and Anderson JM**. Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities. *Am J Physiol Renal Physiol* 291: F1288-1299, 2006.
377. **Vankeerberghen A, Cuppens H, and Cassiman JJ**. The cystic fibrosis transmembrane conductance regulator: an intriguing protein with pleiotropic functions. *J Cyst Fibros* 1: 13-29, 2002.
378. **Verdugo P**. Supramolecular dynamics of mucus. *Cold Spring Harb Perspect Med* 2: 2012.
379. **Verkman AS**. Aquaporins at a glance. *J Cell Sci* 124: 2107-2112, 2011.
380. **Verkman AS, and Mitra AK**. Structure and function of aquaporin water channels. *Am J Physiol Renal Physiol* 278: F13-28, 2000.
381. **Verkman AS, Song Y, and Thiagarajah JR**. Role of airway surface liquid and submucosal glands in cystic fibrosis lung disease. *Am J Physiol Cell Physiol* 284: C2-15, 2003.
382. **Verkman AS, Synder D, Tradtrantip L, Thiagarajah JR, and Anderson MO**. CFTR inhibitors. *Curr Pharm Des* 19: 3529-3541, 2013.
383. **Wadstrom T, and Hisatsune K**. Bacteriolytic enzymes from Staphylococcus aureus. Specificity of ction of endo-beta-N-acetylglucosaminidase. *Biochem J* 120: 735-744, 1970.
384. **Wakida N, Kitamura K, Tuyen DG, Maekawa A, Miyoshi T, Adachi M, Shiraishi N, Ko T, Ha V, Nonoguchi H, and Tomita K**. Inhibition of prostasin-induced ENaC activities by PN-1 and regulation of PN-1 expression by TGF-beta1 and aldosterone. *Kidney Int* 70: 1432-1438, 2006.
385. **Walters MS, Gomi K, Ashbridge B, Moore MA, Arbelaez V, Heldrich J, Ding BS, Rafii S, Staudt MR, and Crystal RG**. Generation of a human airway epithelium derived basal cell line with multipotent differentiation capacity. *Respir Res* 14: 135, 2013.
386. **Walther FJ, David-Cu R, Mehta EI, Polk DH, Jobe AH, and Ikegami M**. Higher lung antioxidant enzyme activity persists after single dose of corticosteroids in preterm lambs. *Am J Physiol* 271: L187-191, 1996.
387. **Wan H, Winton HL, Soeller C, Stewart GA, Thompson PJ, Gruenert DC, Cannell MB, Garrod DR, and Robinson C**. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o. *Eur Respir J* 15: 1058-1068, 2000.
388. **Wang D, Sun Y, Zhang W, and Huang P**. Apical adenosine regulates basolateral Ca2+-activated potassium channels in human airway Calu-3 epithelial cells. *Am J Physiol Cell Physiol* 294: C1443-1453, 2008.
389. **Wang ZW**. Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol Neurobiol* 38: 153-166, 2008.
390. **Watt GB, Ismail NA, Caballero AG, Land SC, and Wilson SM**. Epithelial Na(+) channel activity in human airway epithelial cells: the role of serum and glucocorticoid-inducible kinase 1. *Br J Pharmacol* 166: 1272-1289, 2012.
391. **Westergaard CG, Porsbjerg C, and Backer V**. Emerging corticosteroid agonists for the treatment of asthma. *Expert Opin Emerg Drugs* 20: 653-662, 2015.

392. **Whitcutt MJ, Adler KB, and Wu R**. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In Vitro Cell Dev Biol* 24: 420-428, 1988.
393. **Whitt JP, Montgomery JR, and Meredith AL**. BK channel inactivation gates daytime excitability in the circadian clock. *Nat Commun* 7: 10837, 2016.
394. **Wong LB, and Yeates DB**. Luminal purinergic regulatory mechanisms of tracheal ciliary beat frequency. *Am J Respir Cell Mol Biol* 7: 447-454, 1992.
395. **Wu WL, So SC, Sun YP, Chung YW, Grima J, Wong PY, Yan YC, and Chan HC**. Functional expression of P2U receptors in rat spermatogenic cells: dual modulation of a Ca(2+)-activated K+ channel. *Biochem Biophys Res Commun* 248: 728-732, 1998.
396. **Wyatt TA, Spurzem JR, May K, and Sisson JH**. Regulation of ciliary beat frequency by both PKA and PKG in bovine airway epithelial cells. *Am J Physiol* 275: L827-835, 1998.
397. **Xu X, Browning VL, and Odorico JS**. Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells. *Mech Dev* 128: 412-427, 2011.
398. **Yan J, and Aldrich RW**. LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. *Nature* 466: 513-516, 2010.
399. **Yan J, Olsen JV, Park KS, Li W, Bildl W, Schulte U, Aldrich RW, Fakler B, and Trimmer JS**. Profiling the phospho-status of the BKCa channel alpha subunit in rat brain reveals unexpected patterns and complexity. *Mol Cell Proteomics* 7: 2188-2198, 2008.
400. **Yanez-Mo M, Barreiro O, Gordon-Alonso M, Sala-Valdes M, and Sanchez-Madrid F**. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. *Trends Cell Biol* 19: 434-446, 2009.
401. **Yang B, and Verkman AS**. Water and glycerol permeabilities of aquaporins 1-5 and MIP determined quantitatively by expression of epitope-tagged constructs in Xenopus oocytes. *J Biol Chem* 272: 16140-16146, 1997.
402. **Yang D, Chen Q, Hoover DM, Staley P, Tucker KD, Lubkowski J, and Oppenheim JJ**. Many chemokines including CCL20/MIP-3alpha display antimicrobial activity. *J Leukoc Biol* 74: 448-455, 2003.
403. **Yeo NK, and Jang YJ**. Rhinovirus infection-induced alteration of tight junction and adherens junction components in human nasal epithelial cells. *Laryngoscope* 120: 346-352, 2010.
404. **Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, and Corrigan C**. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 174: 8183-8190, 2005.
405. **Yoon JH, Koo JS, Norford D, Guzman K, Gray T, and Nettesheim P**. Lysozyme expression during metaplastic squamous differentiation of retinoic acid-deficient human tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* 20: 573-581, 1999.

406. **Yoshida M, Nakayama K, Yasuda H, Kubo H, Kuwano K, Arai H, and Yamaya M**. Carbocisteine inhibits oxidant-induced apoptosis in cultured human airway epithelial cells. *Respirology* 14: 1027-1034, 2009.
407. **You Y, Huang T, Richer EJ, Schmidt JE, Zabner J, Borok Z, and Brody SL**. Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 286: L650-657, 2004.
408. **Zaidman NA, Panoskaltsis-Mortari A, and O'Grady SM**. Differentiation of human bronchial epithelial cells: role of hydrocortisone in development of ion transport pathways involved in mucociliary clearance. *Am J Physiol Cell Physiol* 311: C225-236, 2016.
409. **Zeissig S, Fromm A, Mankertz J, Zeitz M, Fromm M, and Schulzke JD**. Restoration of ENaC expression by glucocorticoid receptor transfection in human HT-29/B6 colon cells. *Biochem Biophys Res Commun* 344: 1065-1070, 2006.
410. **Zhang J, and Yan J**. Regulation of BK channels by auxiliary gamma subunits. *Front Physiol* 5: 401, 2014.
411. **Zhang L, Peeples ME, Boucher RC, Collins PL, and Pickles RJ**. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. *J Virol* 76: 5654-5666, 2002.
412. **Zhao KQ, Xiong G, Wilber M, Cohen NA, and Kreindler JL**. A role for two-pore K(+) channels in modulating Na(+) absorption and Cl(-) secretion in normal human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 302: L4-L12, 2012.
413. **Zhou R, Patel SV, and Snyder PM**. Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC. *J Biol Chem* 282: 20207-20212, 2007.
414. **Zhou X, Wulfsen I, Korth M, McClafferty H, Lukowski R, Shipston MJ, Ruth P, Dobrev D, and Wieland T**. Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C. *J Biol Chem* 287: 32161-32171, 2012.
415. **Zhou XB, Wulfsen I, Utku E, Sausbier U, Sausbier M, Wieland T, Ruth P, and Korth M**. Dual role of protein kinase C on BK channel regulation. *Proc Natl Acad Sci U S A* 107: 8005-8010, 2010.
416. **Zhou Y, and Lingle CJ**. Paxilline inhibits BK channels by an almost exclusively closed-channel block mechanism. *J Gen Physiol* 144: 415-440, 2014.
417. **Zhu L, Lee PK, Lee WM, Zhao Y, Yu D, and Chen Y**. Rhinovirus-induced major airway mucin production involves a novel TLR3-EGFR-dependent pathway. *Am J Respir Cell Mol Biol* 40: 610-619, 2009.
418. **Zhu S, Browning DD, White RE, Fulton D, and Barman SA**. Mutation of protein kinase C phosphorylation site S1076 on alpha-subunits affects BK(Ca) channel activity in HEK-293 cells. *Am J Physiol Lung Cell Mol Physiol* 297: L758-766, 2009.