Adrenergic Regulation of CFTR-Dependent Anion Secretion and Cell Migration in Airway Epithelial Cells

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Abstract

The overall goal of this thesis was to understand the mechanisms underlying β-adrenergic receptor (β-AR) regulation of anion secretion and epithelial cell migration on mucociliary clearance and epithelial repair in human airways. The first chapter of the thesis presents an overview of the literature with the purpose of providing background information of mucociliary clearance, airway epithelial cell restitution and specific inflammatory disease conditions where these processes contribute to the innate defense of the airways. Chapters 2 and 3 describe the methods, results and a discussion of experiments that were performed to address the overall goal of the thesis. The final chapter presents a general discussion of the major findings of these studies in the context of what is presently known about the role of β-AR regulation of epithelial function in airways.

Initial experiments presented in Chapter 2 so looked at the effects of growth conditions on β-AR localization in a human airway epithelial cell model system (Calu-3 cells). The results from these experiments showed that under air-liquid interface (ALI) conditions basolateral stimulation with 1μM epinephrine produced a significant increase in CFTR dependent anion secretion, whereas cells that were grown under liquid liquid interface (LLI) had a much smaller increase secretion when stimulated with the same concentration of epinephrine. These results indicated that basolateral expression of β-ARs is increased when cells were grown under ALI. Furthermore Calu-3 cells that were apically

stimulated with the β_2-AR selective agonist salbutamol produced an increase in anion secretion similar to what was observed with 8cpt-cAMP, a non-metabolizable analog of cyclic AMP. Additionally, when cells were treated with carvedilol, an inverse agonist acting at β_2-ARs, an initial decrease in basal Isc occurred and carvedilol treatment after stimulation with 8cpt-cAMP inhibited anion secretion. Cells pretreated with nocotazole, an agent that disrupts microtubule assembly, blocked the inhibitory effects of carvedilol on anion secretion, suggesting that endocytosis was necessary in order to observe the inhibitory effects of carvedilol. Finally, western blot analysis of apical membrane proteins showed that when cells were treated with carvedilol there was reduced expression of CFTR in the apical membrane, which was not observed following stimulation with epinephrine.

Experiments in chapter 3 were designed to investigate the effects of β-AR agonists on epithelial cell migration. Stimulation of Normal Human Bronchial Epithelial (NHBE) cells and Calu-3 cells), with a β_2-AR agonist produced a significant increase in time to wound closure compared to untreated control cells. Moreover, agonist stimulated cells were rescued when pretreated with β-AR antagonists propranolol or ICI-118551. The addition of β-AR agonists epinephrine or salbutamol to CFTR silenced cells (shCFTR) or cells where CFTR was inhibited with 20μM $CFTR_{inh}$-172 showed no further decrease in migration rate suggesting that inhibition due to a change in CFTR expression or activity. Furthermore β-AR agonists reduced lamellipodia protrusion similar to what was

observed after CFTR inhibition. Overall these results suggest that treatment of airway epithelial cells with β-AR agonists causes a decrease in migration rate leading to a significant reduction in the time required for complete wound closure. Additionally these results suggest that stimulating cells with carvedilol causes inhibition of cAMP-stimulated anion secretion by promoting retrieval and internalization of CFTR from the apical membrane. These results signify that the chronic use of β-AR agonists can lead to increased risk for infection in individuals with obstructive airway disease and the use of bias ligands that promote G_s signaling while blocking β-arrestin signaling may be the key to effectively treating these patients. These drugs would potentially promote wound repair and mucociliary clearance while limiting the risk of increased exacerbations and infection

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Chapter 1

Introduction

A. Introduction

1. Anatomical Considerations

The respiratory system is composed of upper and lower airways that make up most of the dead space volume within the lung. The upper airways include the nose, mouth, pharynx and larynx, which serve to warm and moisten the inhaled air before reaching the trachea (Martin, Frija, Burgel 2013; Strohl, Butler, Malhotra 2012) The lower airways are composed of the trachea, bronchi, bronchioles and alveoli (Martin, Frija, Burgel 2013; Strohl, Butler, Malhotra 2012). Both components are important in the process of respiration, however in this thesis the focus will be on the lower airways. The lower airways can be categorized into two zones. The conducting zone, consisting of the trachea bronchi and bronchioles, and the respiratory zone made up of the alveoli (Rock 2014). The epithelium of the conducting airways is essential in removal of inspired foreign material, wound repair and innate immune defense against microbial infection. The major cell types that make up the epithelium within the conducting airways are basal cells, ciliated cells, goblet cells, Clara cells and neuroendocrine cells which form a pseudostratified epithelium (Hackett et al. 2011; Hajj et al. 2007a; Hajj et al. 2007b; Rackley and Stripp 2012; Roomans 2010; Yang and Chen 2014). There are two main cells types that have the ability to differentiate and repair the epithelium after epithelial damage. These cells are basal cells and Clara cells (Crystal et al. 2008; Rackley and Stripp 2012). Basal cells are progenitor cells that can differentiate into both ciliated and goblet cells

where Clara cells are secretory cells that can differentiate into ciliated cells or divide to form new Clara cells (Rackley and Stripp 2012). As one moves down the airways, the number of goblet cells declines and Clara cells become more dominant. This is one of the landmarks for the transition from cartilaginous upper bronchi to the terminal distal bronchioles (Crystal et al. 2008; Davis and Dickey 2008; Rackley and Stripp 2012).

The respiratory zone of the lower airways is primarily responsible for gas exchange. The major epithelial cell types located in this region include Clara cells, alveolar type I and alveolar type II cells (Matthay, Robriquet, Fang 2005; Rackley and Stripp 2012). Alveolar type I cells are responsible for the gas exchange process and also play a role in alveolar fluid reabsorption (Matthay, Robriquet, Fang 2005; Planes et al. 2010; Rawlins et al. 2008; Rawlins 2008; Sartori and Matthay 2002; Shabbir et al. 2013). The process of fluid reabsorption in the alveoli is important in new borns as well as individuals with edema (Planes et al. 2010; Sartori and Matthay 2002). This process is facilitated by Na^+ uptake in the alveoli, the major player being amiloride senstive Na^+ channel ENaC located in apical membrane (Fronius 2013; Planes et al. 2010; Shabbir et al. 2013). The transport of Na^+ creates a driving force for water to follow, causing excess fluid in the alveolar space to be reabsorbed (Planes et al. 2010; Sartori and Matthay 2002; Shabbir et al. 2013; Vadasz, Raviv, Sznajder 2007). This is accomplished by both ENac and the basoloateral Na^+/K^+ ATPase, which pumps out 3 Na^+ for 2 K^+ ions, together ENaC and Na^+/K^+ ATPase create the needed driving force to allow fluid to be reabsorbed in the alveoli (Fronius 2013; Vadasz,

Raviv, Sznajder 2007). Alveolar type II cells are known to secrete surfactant as well as transport Na^+ and Cl^- out of the alveolar lumen, producing a driving force for fluid absorption. This helps to eliminate fluid within the alveolar space, which occurs as a result of the hydrostatic pressure within the alveolar capillaries and under conditions of lung injury (Matthay, Robriquet, Fang 2005).The alveolar type II cells are also thought to be the progenitor cells in the lower lung giving rise to both alveolar type I cells and alveolar type II cells (Rackley and Stripp 2012).

2. Airway Diseases

Cystic Fibrosis (CF) is a lethal congenital obstructive airway disease that affects 60 million people worldwide, primarily those of European decent (Guggino and Stanton 2006; O'Sullivan and Freedman 2009).This disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that is located on chromosome 7, producing alterations in CFTR structure that lead to disruptions in Cl^- channel function (Boucher 2004; Horsley and Siddiqui 2014; O'Sullivan and Freedman 2009). The most common mutation is the ΔF508 where the phenylalanine residue at position 508 is deleted. This mutation causes misfolding of the CFTR protein, preventing it from trafficking from the endoplasmic reticulum to the apical membrane (Boucher 2004; Guggino and Stanton 2006; Mall et al. 2004). At this time there are more than 1500 known mutations in CFTR, many of which are not fully understood in the context of disease severity (O'Sullivan and Freedman 2009). CF affects multiple body systems including: reproductive, gastrointestinal, endocrine and respiratory

systems. However respiratory disease is the most life threatening, leading to infections in the airways at an early age that persist throughout life (Guggino and Stanton 2006; Horsley and Siddiqui 2014; O'Sullivan and Freedman 2009).

CFTR is an essential ion channel responsible for Cl^- secretion. In addition there is evidence that it also regulates the function of epithelial Na^+ channels (ENaC) that are also localized in the apical membrane of airway epithelial cells. (Chmiel and Davis 2003; Guggino and Stanton 2006). In CF patients where CFTR is either not functioning or is not present in the apical membrane, an increase in ENaC-dependent Na^+ absorption occurs which leads to a decrease in the level of fluid on the airway surface. This reduction in the pericilliary liquid layer (PCL) reduces mucociliary clearance, a process responsible for the transportation of mucus and entrapped foreign material out of the airways (Boucher 2004; Chmiel and Davis 2003; Grasemann and Ratjen 2013; Guggino and Stanton 2006; Horsley and Siddiqui 2014). The decrease in fluid secretion coupled with increased Na^+ and fluid absorption alters the consistency of mucus, causing it to become dehydrated with increased viscosity and greater adherence to the airway surface. (Berdiev, Qadri, Benos 2009). A diminished PCL causes ciliary dysfunction, preventing the cilia from beating properly. This prevents the movement of mucus out of the lung, leading to mucus plugs, which cause airway obstruction, as well as increasing the risk of infection and airway disease (Boucher 2004; Chmiel and Davis 2003; Horsley and Siddiqui 2014). A more detailed discussion of the cellular and molecular mechanisms that underlie mucociliary clearance is presented later in this review.

Bronchodilators mainly β_2-AR agonists are a form of therapy in individuals with CF often used alongside chest physiotherapy (Hordvik et al. 1999; Salvatore and D'Andria 2002). These drugs help relieve symptoms such as wheezing and shortness of breath and aid in the clearing of airway obstructions created from excess mucus build up and epithelial cell damage (Halfhide, Evans, Couriel 2005; Hordvik et al. 1999; Salvatore and D'Andria 2002). Although the use of β_2-AR agonists remains controversial there have been several studies have shown that they can be beneficial for CF patients (Cropp 1996; Halfhide, Evans, Couriel 2005; Hordvik et al. 1999; Kent et al. 2014; Salvatore and D'Andria 2002). It has been shown that both short acting (albuterol) and long acting (salmeterol) β_2-AR agonists help to increase lung function (Halfhide, Evans, Couriel 2005; Kent et al. 2014; Salvatore and D'Andria 2002). These agents promote the dilation of the airways by acting on smooth muscle and enhancing mucociliary clearance by increasing ciliary beat frequency of the airway epithelium (Bennett 2002; Cazzola and Matera 2014; Cropp 1996; Salathe 2002; Ulrik 2014). Both of these mechanisms help to increase airflow and reduce obstructions in the airway allowing for symptom relief (Halfhide, Evans, Couriel 2005).

Inflammation occurs early in patients with CF leading to increased risk to infection as well as future airway damage (Balfour-Lynn and Welch 2012; Chmiel and Konstan 2007; Nichols, Konstan, Chmiel 2008). Glucocorticoids delivered as inhaled corticosteroids (ICS), are a form of therapy used in CF patients to decrease inflammation, mucus production, edema formation and bronchial

hyperactivity (Balfour-Lynn and Welch 2012; Chmiel and Konstan 2007; Nichols, Konstan, Chmiel 2008). Glucocorticoids have also been shown to slow the progress of CF disease on the airways, especially in children treated with these medications (Balfour-Lynn and Welch 2012; Cheng, Ashby, Smyth 2013). The use of glucocorticoids can lead to a decrease in airway inflammation in these patients, however improvements in overall lung function are often minimal and further research is needed (Balfour-Lynn and Welch 2012). The chronic use of glucocorticoids does not come without risk and can lead to negative effects on growth, metabolic disease and osteoporosis (Chmiel and Konstan 2007; Nichols, Konstan, Chmiel 2008).

Chronic Obstructive Pulmonary Disease (COPD) is another common airway disease that is characterized by airway obstruction produced by chronic inflammation leading to increased dyspnea, coughing and sputum production over time (Aaron 2014; Qureshi, Sharafkhaneh, Hanania 2014). COPD affects about 24 million people in the United States and about 120,000 deaths per year (Qureshi, Sharafkhaneh, Hanania 2014). People that suffer from COPD have chronic inflammation and are highly sensitive to viral, bacterial and other particulates from the environment that lead to exacerbation of symptoms (Qureshi, Sharafkhaneh, Hanania 2014). These exacerbations trigger an immune response which results in increased mucus production and emphysema onset (Aaron 2014; Martin, Frija, Burgel 2013). People with COPD suffer damage to the small airways which are due to an abnormal epithelium caused by repeated

encounters with cigarette smoke, dust, or fumes and the need for the airway epithelium to repair itself (Martin, Frija, Burgel 2013; Qureshi, Sharafkhaneh, Hanania 2014; Rab et al. 2013). This damaged epithelium leads to narrowed airways and increased mucus both contributing to the obstructive nature of this disease (Qureshi, Sharafkhaneh, Hanania 2014). Furthermore the onset of emphysema in COPD patients is thought to be due to the continual infammatory episodes that are attributed to this disease (Aaron 2014; Martin, Frija, Burgel 2013). These exacerbations lead to terminal bronchiole destruction causing decreased support and the collapse of alveolar walls (Martin, Frija, Burgel 2013).

Persons that suffer from COPD have similar airway problems as those that suffer from CF; these include defects in mucocilary clearance which include decreased PCL inhibiting ciliary movement and leading to decreased clearance of debris, bacteria and other foreign materials in the airways (Cantin et al. 2006; Rab et al. 2013). This leads to increased risk of infection (Rab et al. 2013). Furthermore COPD leads to increased inflammatory response due to higher rate of infection in the lungs similar to what is seen in CF (Rab et al. 2013). All of these symptoms in COPD are due to the loss of CFTR function, unlike CF patients that have limited CFTR function or limited CFTR in the membrane (Rab et al. 2013). The loss of CFTR function in COPD is thought to be due to the inhalation of toxic chemicals like cigarette smoke which have shown to have a negative impact on anion secretion in the airways, which is essential for proper lung function and the ability to maintain a healthy airway (Cantin et al. 2006; Rab et al. 2013).

Individuals with COPD take bronchiodialators to help open the airways to allow for maximum airflow (Baker et al. 2014; Qureshi, Sharafkhaneh, Hanania 2014). Short acting β_2-AR agonists like albuterol are used in these patients to help control exacerbations (Qureshi, Sharafkhaneh, Hanania 2014). However the use of long acting β_2-AR agonists like formoterol or salmeterol have also been shown to decrease exacerbations (Baker et al. 2014; Nardini et al. 2014; Qureshi, Sharafkhaneh, Hanania 2014). These drugs can produce significant improvement in COPD patients that take them, increasing tolerance of physical activity as well as over all lung function (Nardini et al. 2014). Although β_2-agonists can be used alone to help with symptom relief, studies have shown pairing them with ICS improves the function of the airways in a COPD patient as opposed to either used alone (Kew, Dias, Cates 2014; Miller-Larsson and Selroos 2006).

The use of glucocorticoids in patients with COPD aid in decreasing the inflammation of the airways as well as mucus production (Ammit 2013; Balfour-Lynn and Welch 2012; Boardman et al. 2014; Kew, Dias, Cates 2014; Nichols, Konstan, Chmiel 2008). These drugs are more often times paired with β_2-AR agonists, however individuals with chronic COPD may take them as a monotherapy due to increased inflammation from recurrent epithelial damage (Ammit 2013). The use of these drugs in combination therapies allows for both aspects of the disease, bronchoconstriction and inflammation, to be targeted and corrected, evidence suggests they increase the ability for the ICS to reduce

inflammation (Chung, Caramori, Adcock 2009; Mansfield 2008; Miller-Larsson and Selroos 2006).

Asthma represents another obstructive airway disease that affects the lives of roughly 8% of adults and more than 9% of children living in the United States (Centers for Disease Control and Prevention (CDC) 2011; Moorman et al. 2012). Asthmatics suffer from symptoms including chest constriction, shortness of breath, mucus over production, and excess coughing and wheezing (Erle and Sheppard 2014; Moorman et al. 2012; Walker et al. 2011). These symptoms are brought on by airborne irritants such as pollution, allergens, chemicals and other inhaled material which lead to bronchioconstriction and inflammation. Over time persistent asthma attacks may lead to airway remodeling (Elias et al. 1999; Erle and Sheppard 2014; Moorman et al. 2012). Airway remodeling occurs in response to chronic epithelial injury which ultimately results in airway wall thickening, increase in mucus secretion and changes in epithelial cell morphology including possible ciliary dysfunction (Erle and Sheppard 2014; Wasilewski, Lougheed, Fisher 2014). There is no cure for asthma but there are therapeutic approaches that help relieve the symptoms associated with this disease (Lipworth 2007; Moorman et al. 2012).

β_2-adrenergic receptor agonists are typically used to control asthma symptoms because of their ability to induce airway smooth muscle relaxation, promote mucus clearance and increase airflow (Erle and Sheppard 2014; Lipworth 2007; Walker et al. 2011). β_2-AR agonists are the recommended

treatment and a standard for treating asthmatics (Bonini et al. 2013; Clifford and Knox 2012). The use of short acting β-agonist drugs like albuterol are the most common form of treatment in asthma (Xia et al. 2013). These drugs are mainly used by individuals with intermittent asthma and are to be used occasionally when an asthmatic episode occurs (Xia et al. 2013) however long acting β_2-agonists are also used in people that suffer from asthma. These drugs are prescribed to help in the maintenance of those who have recurrent episodes of wheezing, shortness of breath or chest tightness (Cates et al. 2014; Saguil and Garcia 2014). Long acting β_2-agonists are meant to prevent asthmatic episodes from occurring and if one were to occur it is thought use of these drugs would decrease the intensity and length of the episode (Crompton 2006; Mak and Hanania 2012).

Glucocorticoids are another type of medication that are used to control asthma. These drugs are often used as a controller medication, reducing airway inflammation (Mansfield 2008). Gluocorticoids have been a standard in asthma treatment (Boardman et al. 2014) for short periods of time, this is due to the side effects these drugs can cause (Vandevyver et al. 2013). Like β_2-agonists these drugs are usually taken through an inhaler or nebulizer (Crompton 2006). Glucocorticoids are often paired with β_2-AR agonist providing increased symptom relief for the individuals that take them (Mansfield 2008; Miller-Larsson and Selroos 2006). The individual use of glucocorticoids are a less effective therapy

in asthma treatment and have major side effects (Barnes and Adcock 2009; Crompton 2006; Newton 2014; Roos and Nord 2012).

3. Airway Disease Therapuetics

A common therapeutic strategy for the treatment of CF, COPD and asthma is the use of inhaled β_2 receptor agonists and glucocorticoids to alleviate acute airway responses to airborne agents that can exacerbate inflammation and to minimize immunologic responses that can lead to long term damage to the airways. The following sections provide a brief review of the receptors and signaling mechanisms associated with the airway epithelium that mediate the response to these important therapeutic agents.

a. Adrenergic receptor expression and signaling:

Adrenergic receptors expressed in the lung include both alpha (α) and beta (β) G-protein coupled receptor (GPCR) subtypes. Airway smooth muscle cells possess α_1 and α_2 subtypes (Barnes 1989; Goldie, Paterson, Lulich 1990). Stimulation of these receptors causes smooth muscle contraction that reduces airway caliber and airflow, however they do so through two different mechanisms (Goldie, Paterson, Lulich 1990). Agonist binding to theα_1 receptor causes calcium release through $G_{q/11}$ signaling (α_{1b} receptors) or through the activation of nifedipine-sensitive Ca^{2+} channels resulting in Ca^{2+} uptake from the extracellular media (a_{1a} receptors). In contrast, α_2 receptors are coupled to the G_i signaling cascade, causing a decrease in adenylyl cyclase activation and a

reduction in cyclic AMP (cAMP) (Goldie, Paterson, Lulich 1990). β-receptors in the lung are expressed by smooth muscle cells, airway epithelial cells, sensory nerves, alveolar epithelial cells and immune cells (Barnes 2004). β_1 and β_2 subtypes have been identified and have been shown to signal through G_s leading to the production of cAMP and activation of protein kinase A (PKA). β_2 receptors are the most highly expressed in the airways (Barnes 2004). When stimulated β_2 receptors cause smooth muscle relaxation and increase airflow into the lung. They also increase ciliary beat frequency of ciliated epithelial cells and an increase in electrolyte, fluid and mucus secretion from submucosal gland epithelial cells and clara cells (Barnes 2004; Goldie, Paterson, Lulich 1990; Shore and Moore 2003). Coupling of β_2 adrenergic receptors to adenylyl cyclase and subsequent increases in cAMP is critical for producing the therapeutic benefits of β_2 agonists in diseases such as CF, COPD and asthma (Bennett 2002). The combined effects of bronchorelaxation and increased epithelial fluid secretion and ciliary beating serves to reduce airway obstruction and enhance the clearance of mucus and potentially infectious microorganisms from the lung (Barnes 1995; Barnes 2004; Bennett 2002; Goldie, Paterson, Lulich 1990; Shore and Moore 2003).

As previously discussed the use of β_2-AR in obstructive airway disease is a common form of therapy (Halfhide, Evans, Couriel 2005; Kent et al. 2014; Salvatore and D'Andria 2002). Common β_2-agonists that are used are short acting agonists, albuterol or salbutamol and long acting agonists, salmeterol and

formeterol, all of which are used in CF, COPD and asthma (Halfhide, Evans, Couriel 2005; Kent et al. 2014; Salvatore and D'Andria 2002). Short acting agonists are mainly used for fast symptom relief. During an asthma attack or when individuals are having increased difficulty breathing (Paris et al. 2008; Qureshi, Sharafkhaneh, Hanania 2014; Xia et al. 2013). The use of long acting β-agonists is becoming more common in patients with these diseases. These medications like salmeterol are usually inhaled through a nebulizer and last up to 12 hours. This allows prolonged symptom relief and is thought to decrease exacerbations that would warrant the use of short acting agonists (Baker et al. 2014; Cates et al. 2014; Nardini et al. 2014; Qureshi, Sharafkhaneh, Hanania 2014; Saguil and Garcia 2014; Tan and Corren 2014).

The popularity of these β-AR therapeutics is unfortunately associated with negative consequences. The chronic use of β_2-agonists leads to desensitization of airway smooth muscle cells in the response to a β_2-agonist (Clifford and Knox 2012). This leads to decreased effectiveness of these medications in dilating the airways for maximal airflow (Clifford and Knox 2012). This desensitization is mainly due to the down-regulation of the β_2-AR receptor leading to a reduction in available agonist binding sites and therefore, subsequent activation of G-protein signaling. (Clifford and Knox 2012). This phenomenon occurs through the activation of β-arrestin signaling pathway, a second pathway stimulated by the binding of a β-agonist to the β_2-AR. The effects of this pathway are different between the β_1-AR and β_2-AR where β-arrestin has higher affinity to the β_2-AR

verses β_1-AR (Kobayashi et al. 2005; Reiter and Lefkowitz 2006; Shenoy et al. 2006). When β_2-ARs are stimulated, G-protein receptor kinases (GRKs) are activated and recruited to phosphorylate the C-terminal tail of the receptor (Ma and Pei 2007; Reiter and Lefkowitz 2006; Tohgo et al. 2003). The phosphorylated tail of the GPCR recruits and activates β-arrestins leading to downstream signaling of desensitization (Reiter and Lefkowitz 2006; Shenoy et al. 2006). Once β-arrestin is recruited it causes uncoupling of the GPCR by binding to the C-terminal tail deactivating the receptor (Kobayashi et al. 2005; Ma and Pei 2007; Reiter and Lefkowitz 2006; Tohgo et al. 2003). β-arrestin will then bind clathrin which will promote the internalization process of the receptor through endocytosis via clathrin coated pits, promoting the desensitization process of the receptor (Ma and Pei 2007; Shenoy et al. 2006; Tohgo et al. 2003). It has also been shown that β-arrestin acts as a scaffold for MAPK by binding ERK1/2 allowing further downstream signaling in a G-Protein independent manner (Kobayashi et al. 2005; Reiter and Lefkowitz 2006; Shenoy et al. 2006; Tohgo et al. 2003). It is the combination of all these effects that cause the β_2-AR desensitization.

A potential problem with certain agonists is that the receptors that are activated can signal through more than one pathway leading to undesirable side effects which often limit their therapeutic usefulness (Tilley 2011). Studies over the past decade have shown that structural modifications of these agonists can induce distinct conformational changes in the receptor. These changes can bias

signaling towards one pathway relative to the others so that activation of downstream targets that cause detrimental side effects can potentially be minimized (Andresen 2011; Tilley 2011). The use of bias ligands for treating various conditions is a fairly recent development. A bias ligand will bind to a GPCR, for example and activate one of two or more signaling pathways, such as $G\alpha\beta\gamma$, while reducing the activation of the β-arrestin pathway or vice versa, reducing the activation of $G\alpha\beta\gamma$ while increasing β-arrestin signaling (Andresen 2011; Strachan et al. 2014; Tilley 2011). One studied model of bias signaling is with the angiotension II type 1 receptor (AT1R). This GPCR promotes G_q activation leading to Ca^{2+} release and is a target for antagonists in the treatment of hypertension and heart failure (Tilley 2011). When a bias ligand such as TRV120023 (Kim et al. 2012; Monasky et al. 2013; Strachan et al. 2014; Tilley 2011; Violin et al. 2010) binds to AT1R a conformational change in the receptor occurs promoting β-arrestin signaling (Andresen 2011; Monasky et al. 2013; Strachan et al. 2014; Tilley 2011). This bias ligand produces an increased response to β-arrestin signalling while having an equal amount of decreased efficacy to G_q signalling (Strachan et al. 2014; Tilley 2011). The activation of β-arrestin signaling has been shown to be beneficial in the treatment of hypertension and heart failure (Kim et al. 2012; Monasky et al. 2013; Tilley 2011). One way is by blocking G_q signaling leading to a decrease in Ca^{2+} release, which has shown to contribute to heart failure and hypertension (Kim et al. 2012; Monasky et al. 2013; Tilley 2011). This is done by promoting

desensitization of the receptor by internalization via clathrin vesicals as well as recrutiment of c-Src and AP-2 which play a part in receptor endocytosis. Furthermore it has been shown that β-arrestin can increase cardioprotective elements, potentially by upregulating MAPK signaling which has been shown to reduce apoptosis in cardiac myocytes (Kim et al. 2012; Tilley 2011). TRV120023 has also shown to increase cardiomyocyte contractility although the exact mechanism on how this effect is produced is still unclear (Strachan et al. 2014; Tilley 2011). Evidence however shows that this may be due to the ligands effects on dephosphorylation of certain proteins which lead to an increase in myofilaments response to Ca^{2+} (Monasky et al. 2013).

Another receptor that has been studied using bias agonism is the 1 parathyroid hormone receptor (PTH1R) which plays an important role in bone formation and kidney function (Appleton et al. 2013; Gesty-Palmer and Luttrell 2011). This is a GPCR that activates G_s and G_q signaling. The activation of PTH1R produces bone formation by stimulating osteoblasts as well as causing bone reabsorption via the activation of osteoclasts (Bohinc and Gesty-Palmer 2013; Ferrari and Bouxsein 2009; Gesty-Palmer and Luttrell 2011). Agonists of PTH1R are used in medicine for patients with osteoporosis, which is a disease that causes a decreased bone mass causing bones to become weak (Bohinc and Gesty-Palmer 2013; Ferrari and Bouxsein 2009; Gesty-Palmer et al. 2009; Gesty-Palmer and Luttrell 2011). These medications are used because they stimulate bone formation overall, however they do cause bone reabsorption

which poses a problem (Ferrari and Bouxsein 2009). Studies have shown that β-arrestin signaling plays an essential part in the process of net bone formation (Bohinc and Gesty-Palmer 2013; Ferrari and Bouxsein 2009; Gesty-Palmer et al. 2009). The use of β-arrestin-targeting bias ligands on PTH1R mediated effects on bone formation have been studied and have shown to increase bone formation in mice while preventing bone reabsorption, having potential in the treatment or prevention of osteoporosis (Gesty-Palmer and Luttrell 2011). The β-arrestin signaling pathway promotes a net gain in bone formation after PTH1R stimulation with an agonist because it causes inhibition of G-Protein signaling as well as initiating PTH1R desensitization (Gesty-Palmer and Luttrell 2011). β-arrestin bias ligands that bind to the PTH1R are thought to uncouple the PTH bone formation effects from its effects on bone reabsorption, however more research is needed to determine the mechanism of these effects in bone (Bohinc and Gesty-Palmer 2013; Gesty-Palmer and Luttrell 2011).

Although the role of bias ligands on the AT1R and PTH1R for treating diseases such as hypertension and osteoporosis are still being studied, there is a common biased ligand used in the treatment of heart failure. This bias ligand is a β_2-AR ligand called carvedilol. Carvedilol is a β-blocker that prevents the recycling of the receptors (Doughty and White 2007; Keating and Jarvis 2003; Kveiborg et al. 2007). When carvedilol blocks β_2-AR signaling it prevents norepinephrine (NE) release, decreasing β_1-AR activation and allowing for a decrease in heart rate thereby reducing the workload for the cardiac muscle

(Kaye et al. 2001; Kveiborg et al. 2007). Carvedilol also blocks α_1-AR producing vasodilatation, again reducing the work of the heart and decreasing hypertension (Book 2002; Doughty and White 2007; Feuerstein and Ruffolo 1996; Keating and Jarvis 2003; Kveiborg et al. 2007). This ligand also prevents the formation of oxygen radicals and the depletion of antioxidants, which leads to a decrease in inflammation; an effect not seen with other β-blockers (Book 2002; Doughty and White 2007; Kaye et al. 2001; Keating and Jarvis 2003; Kveiborg et al. 2007). Although not everything is known about carvedilol's mechanism(s) of action, the use of it in the treatment of chronic heart failure and hypertension has shown to be highly beneficial (Book 2002; Doughty and White 2007; Kaye et al. 2001; Keating and Jarvis 2003; Kveiborg et al. 2007).

b. Glucocorticoid receptors:

Glucocorticoid receptors are another receptor found in the human airways. These receptors are highly concentrated in lung endothelial and epithelial cells (Barnes 2004). Glucocorticoid receptors help regulate inflammatory gene expression by producing a decrease in inflammatory cells leading to an overall reduction in inflammation (Barnes 2004; Ito, Getting, Charron 2006; Roos and Nord 2012; Schwiebert, Stellato, Schleimer 1996). These receptors are in the nuclear receptor subfamily of transcription factors and are also known as NR3C1(Boardman et al. 2014; Chung, Caramori, Adcock 2009; Vandevyver et al. 2013). This receptor is composed of three domains, a C-terminal ligand binding

domain, DNA binding domain and an N-terminal domain, all three domains play an important role in the signaling of the receptor (Boardman et al. 2014; Nixon, Andrew, Chapman 2013; Vandevyver et al. 2013). When a ligand binds to the C-terminal domain the receptor undergoes a conformational change which causes it to be translocated to the nucleus where it mediates gene regulation (Boardman et al. 2014; Nixon, Andrew, Chapman 2013; Vandevyver et al. 2013). One way this receptor regulates gene expression is by binding to to glucocorticoid response elements (GREs), sites on the promoter region of genes, which initiate recruitment of 'activator' or 'repressor' molecules that target transcription (Ammit 2013; Boardman et al. 2014; Nixon, Andrew, Chapman 2013; Vandevyver et al. 2013). The activators lead to increased gene expression of anti-inflammatory proteins like IL-10, where the repressors lead to down regulation of pro-inflammatory cytokines such as IL-8 (Boardman et al. 2014). The repression of gene expression is also accomplished through a process called transrepression. This is where the glucocorticoid receptor binds directly to a transcription factor such as nuclear factor-κB (NF-κB) (a pro-inflammatory transcription factor), acting like an antagonist which interferes with the activation of NF-κB leading to inhibition of transcription of proinflammatory cytokines like IL-1 (Boardman et al. 2014; Nixon, Andrew, Chapman 2013). The inhibition of transcription can also occur through the inhibition of histone acetylases and recruitment of histone deacetylaces both of which cause negative effects on chromatin (Ammit 2013; Boardman et al. 2014). Through these mechanisms glucocorticoid receptor is

able to reduce inflammation and is utilized to treat diseases such as inflammatory bowel and obstructive airway diseases such as asthma and COPD (Ammit 2013; Boardman et al. 2014).

4. Mucociliary Clearance

Ion transport by the ciliated cells of the airway surface epithelium is essential for establishing appropriate osmotic driving forces for the fluid transport required in the mucociliary clearance process. The airway surface liquid (ASL) consists of two phases: the periciliary liquid phase which ensures the movement of cilia, and the mucus phase that is layered on top of the periciliary liquid which is important in trapping foreign materials such as bacteria, dust, and a variety of airborne allergins (Knowles and Boucher 2002; Rackley and Stripp 2012; Toczylowska-Maminska and Dolowy 2012). The ASL allows the removal of these materials from the lungs through the mouth and esophagus (Rackley and Stripp 2012; Toczylowska-Maminska and Dolowy 2012). Ion transport by the surface epithelium involves the regulation of electrogenic Na^+ absorption and anion secretion. The mechanism of anion transport involves several different ion channels and transporters that function in a coordinated manner to produce Cl^- or HCO_3^-. Secretion in the standard model, Cl^- is loaded into the cell via a $Na^+/K^+/2Cl^-$ (NKCC1) transporter located in the basolateral membrane. Chloride exists the cell by way of CFTR, the cAMP and ATP regulated anion channel that is defective in CF and/or TMEM16a, a 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (DIDS) sensitive anion channel that is activated by increases in

intracellular [Ca^{2+}] (Ballard and Spadafora 2007; Gianotti et al. 2013). The Na^+/K^+ ATPase is located on the basolateral membrane which serves to maintain Na^+ and K^+ ion gradients across the plasma membrane and contributes to the resting membrane potential of the cell. The Na^+/K^+ ATPase brings in $2K^+$ for every $3Na^+$ pumped out of the cell. The K^+ that enters the cell as a result of Na^+/K^+ ATPase and NKCC1 activity is then recycled via specific K^+ channel subtypes such as $K_v7.1$/MiRP2 (KCNQ1/KCNE3) or $K_{Ca}3.1$ (KCNN4) depending on whether anion secretion was stimulated by increases in cAMP or [Ca^{2+}] respectively (Cotton 2000). The increase in basolateral K^+ efflux functions as a mechanism for charge compensation to maintain the driving force for apical secretion and the steady-state [K^+] gradient across the membrane (Bleich and Shan 2007; Cotton 2000; Palmer et al. 2006). The apical efflux of Cl^- along with K^+ exit at the basolateral membrane promotes paracellular Na^+ transport through tight junctions between the epithelial cells, resulting in net transepithelial NaCl secretion under physiological conditions (Frizzell and Hanrahan 2012).

Another aspect of ion transport that is essential for mucociliary clearance is the process of Na^+ absorption. Na^+ transport is primarily dependent on apical ENaC (Al-Alawi et al. 2014; Caci et al. 2009; Gianotti et al. 2013). The Na^+ that is transported across the apical membrane is pumped out of the cell across the basolateral membrane by the Na^+/K^+ ATPase. K^+ ions taken up by the pump are recycled across the basolateral membrane through K^+ channels, which helps to sustain the driving force for Na^+ uptake. Na^+ absorption by this mechanism produces a basolaterally positive transepithelial potential difference which serves

as a driving force for the paracellular movement of anions (primarily Cl^-) from the airway lumen into the extracellular fluid (Gianotti et al. 2013). The absorption of NaCl sets up an important osmotic driving force fluid absorption. A balance in the rates of fluid absorption and secretion sets the depth of the periciliary liquid phase so that it matches the height of the cilia. This ensures that the mucus phase does not compress the cilia and restrict their movement, which occurs when the rate of fluid absorption is enhanced as in CF (Al-Alawi et al. 2014; Caci et al. 2009; Gianotti et al. 2013). A model summarizing the mechanisms of Na^+ absorption and anion secretion is presented in Figure 1.

Figure 1

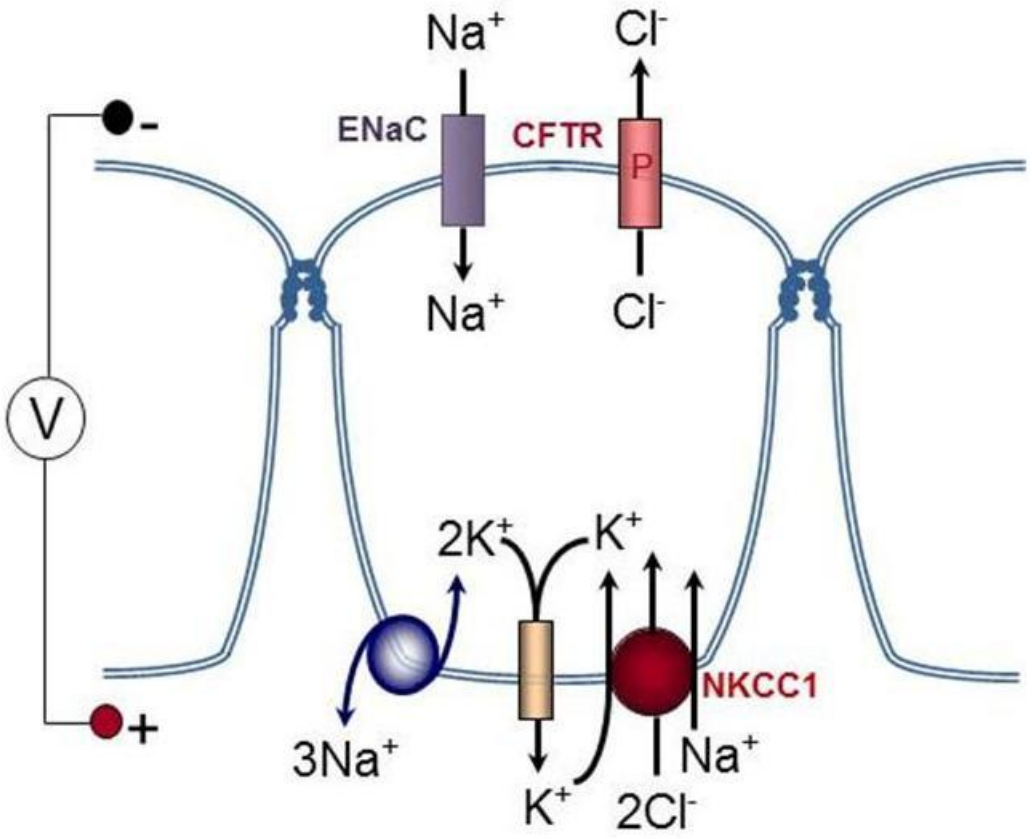

Figure 1: Model illustrating the essential channels needed for the process of mucocilliary clearance in airway epithelial cells. Epithelial Na^+ Channel (ENaC) absorbs Na^+ across the apical membrane which is pumped out of the cell across the basolateral membrane by the Na^+/K^+ ATPase. K^+ ions taken up by the pump are recycled across the basolateral membrane through K^+ channels, which helps to sustain the driving force for Na^+ absorption and Cl^- secretion. The NKCC1 loads K^+, Na^+ and $2Cl^-$ into the cell. The Cl^- exits primarily through CFTR across the apical membrane. It is through the coordinated actions of these ion channels that regulates the depth of the PCL important in facilitating mucociliary clearance.

Na^+ absorption is highly regulated by CFTR (Berdiev, Qadri, Benos 2009; Mall 2008; Reddy and Quinton 2003; Toczylowska-Maminska and Dolowy 2012). When CFTR is activated transport of Na^+ into the cell by ENaC is decreased and

when CFTR is not activated apical Na^+ absorption occurs (Berdiev, Qadri, Benos 2009; Kunzelmann and Mall 2001; Mall 2008; Reddy and Quinton 2003). These mechanisms of fluid secretion and absorption are thought to be due to the coupling behavior of CFTR and ENaC (Berdiev, Qadri, Benos 2009; Varelogianni et al. 2013). Although this coupling behavior is not fully understood it is suggested that CFTR and ENaC are located near each other in the apical membrane potentially forming a complex that allows these two proteins to interact directly (Berdiev, Qadri, Benos 2009).

Cell Migration

The process of cell migration in the airways is another essential process after injury occurs. The process of cell migration involves four main steps; polarization, protrusion, adhesion/traction and finally retraction. This first step of polarization is regulated strongly by chemotaxis elements that allow for the cell to form a leading (front end) and trailing edge (rear end) allowing migration to occur (Ridley et al. 2003; Schwab et al. 2012) This is driven by Rho Family GTPases like division control protein 42 (Cdc42), an essential player in the polarization of cells by contributing to localization of organelles and the microtubule-organizing center (MTOC) (Ridley et al. 2003; Schwab et al. 2012). Polarization of cells promotes cell protrusion at the front of the cell (Ridley et al. 2003; Roca-Cusachs, Sunyer, Trepat 2013). These protrusions are called filapodia which are long thin extensions of the cell consisting of a barbed end and a pointed end forming long bundles made of actin that act as sensors, or lamellipodia which are

larger and wider extensions created by branching polymerized actin filaments that are responsible for the migratory process and forward movement of cells (Ridley et al. 2003; Schwab et al. 2012; Vicente-Manzanares, Webb, Horwitz 2005). During migration cells adhere to the extra cellular matrix to promote satiability and forward movement. This is mediated by myosin II, which interacts with actin filaments creating traction for the migrating cells, and integrin adhesions acting as anchors at the protruding end of cells allowing forward movement (Ridley et al. 2003). The turnover of integrins is essential in the process of migration. After forward movement occurs adhesions reassemble to the ECM, aiding in traction, and disassemble from the trailing end which allows continual forward movements (Ridley et al. 2003; Schwab et al. 2012) The disassembly of actin from the trailing end marks the final step in migration, retraction (Ridley et al. 2003; Vicente-Manzanares, Webb, Horwitz 2005). Myosin II is also involved in rear end detachment of the migrating cells. This is needed to create tension between the integrins/adhesions and the retraction of the tail end of the cell (Ridley et al. 2003; Vicente-Manzanares, Webb, Horwitz 2005). These four steps ensure that the process of wound repair is accomplished and that cells migrate as a single unit to repair the damaged epithelium (Schiller, Maniak, O'Grady 2010; Wang et al. 2003; Zhao et al. 1996a).

Extracellular signal-related kinase (ERK)/ mitogen activate protein kinase (MAPK) are major players in the migratory process of keratinocytes and corneal epithelial cells. Increasing ERK and or MAPK activity promotes cell migration and proliferation (Lu, Reinach, Kao 2001; Pullar, Chen, Isseroff 2003; Wang et al.

2003). However it has been shown that β_2-AR also plays a role in the regulation of cell migration in these cells (Chen, Hoffman, Isseroff 2002; Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003). When β_2-AR is stimulated with an agonist, like epinephrine, the migration rate is significantly reduced, but when stimulated with an antagonist the opposite effect occurs, an increase in the migration rate is observed (Chen, Hoffman, Isseroff 2002; Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003; Pullar et al. 2006; Pullar et al. 2012). It has been shown in subsequent studies that β_2-AR stimulates the activity of protein phosphatase 2A, a serine/threonine phosphatase that can dephosphorylate ERK, therefore reducing the stimulatory effect of ERK on migration (Pullar, Chen, Isseroff 2003; Pullar et al. 2006). In contrast, β_2-AR antagonists prevent PP2a activation thus promoting cell migration. Thus the results from these experiments provided insight as to the identity of the signaling pathway that links β_2-AR activation to a reduction in the rate of cell migration (Ghoghawala et al. 2008; Pullar, Rizzo, Isseroff 2006; Pullar et al. 2007).

Another major contributor to cell migration are guidance cues cells use to aid in the process of migration (Roca-Cusachs, Sunyer, Trepat 2013; Zhao et al. 1996a). These cues involve gradients and forces of the ECM and intercellular matrix. Some of these cues include chemotaxis, the response to a chemical stimulus, mechanotaxis, the response to physical force at cell to cell or cell to ECM level, durotaxis, cells response to ECM stiffness and electrotaxis, the cells response to an electrical field (EF) (Reid and Zhao 2014; Roca-Cusachs, Sunyer,

Trepat 2013; Zhao et al. 1996a). For this study we will focus on the effects of electrotaxis in epithelial cell migration.

Studies done in corneal epithelial cells, keratinocytes and airway epithelial cells have shown that when the epithelium is wounded, a lateral current can be detected that establishes a local electric field (EF) that can be sensed by epithelial cells (Reid and Zhao 2014; Song et al. 2007; Sun et al. 2011; Zhao et al. 1996b). This occurs because the Transepithelial potential (TEP) of the epithelial layer is destroyed due to wounding (Reid and Zhao 2014; Sun et al. 2011). During this process the cells at the wounded edge align themselves perpendicular to the EF and migrate toward the cathode located at the center of the wound (see figure 2). This type of directed migration where the EF serves as a guidance cue is called electrotaxis (Reid and Zhao 2014; Song et al. 2007; Sun et al. 2011; Wang et al. 2003; Zhao et al. 1996a; Zhao et al. 1996b). The EF also contributes to the reorientation of growth factor and chemosensory receptors as well as cytoskeleton elements towards the front (leading edge) of the cells located at the wounds edge, (Zhao et al. 1996a; Zhao et al. 1996b) promoting actin polymerization which facilitates lamellipodia protrusion and cell migration (Song et al. 2002; Zhao et al. 1996a; Zhao et al. 1996b).These changes in the receptor concentration at the leading edge of the cells allow for detection of growth factors or chemotactic molecules which also play an important role in wound repair (Song et al. 2002; Zhao et al. 1996a).

Figure 2

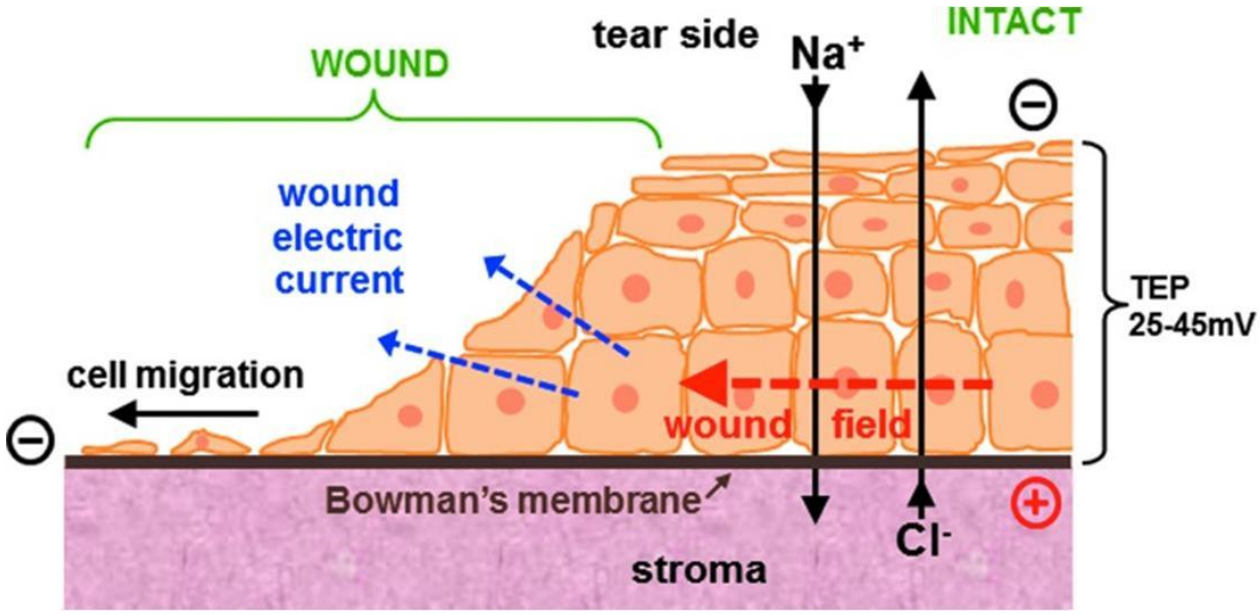

Figure 2: Model of corneal epithelial cells showing damaged epithelial cells produce electrical fields (EFs) which act as a guidance cue for cells to migrate toward the wound center (cathode). Transepithelial Potential (TEP). "Reprinted with permission from ADVANCES IN WOUND CARE 3/2, (184-201) 2014, published by Mary Ann Liebert, Inc., New Rochelle, NY."

Additionally, the role of CFTR in the process of cell migration has been studied and was shown to be an essential component of airway epithelial cell migration. Previous studies showed that CFTR was a major contributor in determining the rate of cell migration (Schiller, Maniak, O'Grady 2010; Sun et al. 2011). This is due to multiple factors which include a role for CFTR in lamellipodia formation (Schiller, Maniak, O'Grady 2010), as well as the importance of CFTR in the generation of an EF following epithelial damage (Sun et al. 2011). Studies by Nguyen et al have shown that CFTR corrector VRT-325 increased the rate of wound closure in cells that expressed the ΔF508 mutation. This increase in wound closure was comparable to what was seen in wt-CFTR

cells. It is thought that the increase of migration in the ΔF508-CFTR cells after CFTR corrector is due to the up-regulation of CFTR in these cells (Trinh et al. 2012). This is additional evidence that CFTR is essential in the process of wound repair in epithelial cells. Another important role of CFTR in the migration process is the effect it has on apical membrane expression of glycosphingolipid GM1. CFTR deficient cells have decreased levels of glycosphingolipid GM1 which plays an important role in β_1-integrin activation, phosphorylation of FAK and p130 Crk- associated substrate (CAS), all of which highly contribute to wound repair efficiency in airway epithelial cells (Itokazu et al. 2014). These results provide compelling evidence that CFTR and Cl^- secretion is critical for the wound repair process.

B. Scope of Thesis

Obstructive airway diseases like CF, asthma and COPD affects millions worldwide and significantly impairs the quality of life of the individuals who suffer from these airway diseases. Many people that have an obstructive airway disease take β_2-agonists to help relieve symptoms such as of shortness of breath and wheezing. However studies have shown that the effects of β_2-agonists on the airway epithelium could have significant negative side effects that could potentially lead to decreased symptom relief as well as increased airway infections (Bhagat, Swystun, Cockcroft 1996; Booth et al. 1996; Ramage et al. 1994; Spina 2014). Although the effects of β_2-AR stimulation on wound repair

have been well studied in keratinocytes and corneal epithelial cells, the effects of their activation on airway epithelial cell migration is poorly understood. The need for further investigation of the effects of β_2-AR agonists on airway epithelial cell migration and overall lung health is important for understanding the underlying cellular and molecular mechanisms involved. A better understanding of these mechanisms may help to direct the course of drug development with improved effectiveness and minimal negative side effects.

Therefore, the overall hypothesis of this thesis is that chronic stimulation of β_2-AR leads to down-regulation coupled to a reduction in CFTR expression and activity in the apical membrane that impairs mucociliary clearance and wound repair.

The overall goal is to understand the mechanism of adrenergic regulation of CFTR function responsible for reductions in mucociliary clearance and airway wound healing. An understanding of this mechanism may aid in the development of new drugs to reduce the possible side effects of long-acting β-agonists on wound healing associated with recurrent episodes of inflammation in patients with obstructive airway disease like asthma and CF.

The first aim of this study is to investigate the effects β-AR agonists have on mucociliary clearance and wound repair.

Hypothesis: Stimulation of β_2-AR with an agonist cause distinct downstream signaling pathways in airway epithelial cells. We propose that these agonists lead

to the activation of β-arrestin signaling which produces inhibition of anion secretion and wound repair through a decrease in CFTR activity.

The second aim of this study is to determine the effects β-AR inverse ligand carvedilol has on mucociliary clearance and wound repair.

Hypothesis: Stimulation of β_2-AR with carvedilol blocks G-protein signaling and activates β-arrestin signaling, leading to a decrease in CFTR activity as well as a decrease in available CFTR in the apical membrane.

The third and final aim of this study was to investigate the functional interactions between CFTR and β_2-AR after β-AR stimulation that accounts for the decrease in CFTR activity.

Hypothesis: Chronic β_2-AR stimulation with an agonist inhibits CFTR activity through PP2a activation while β_2-AR stimulation with inverse ligand carvedilol decreases CFTR activity through internalization and reduced surface expression of the β_2-AR/CFTR signaling complex. These two mechanisms result in reduced CFTR channel activity that is responsible for the decrease in mucociliary clearance and wound repair.

The results of these studies will provide an important understanding of the mechanisms responsible for the inhibitory effects β_2-AR ligands have on mucociliary clearance and migration in airway epithelial cells. This will offer new knowledge of β-AR effects on CFTR which could help reduce the possible negative side effects on mucociliary clearance and wound repair through new β_2-AR druge targets.

Chapter 2

β-Adrenergic Receptor Expression and Inverse Agonist Regulation of Anion Secretion in Human Airway Epithelial Cells

A. Summary

The objective of this study was to investigate the effects of air-liquid (ALI) and liquid-liquid (LLI) interface conditions on membrane localization of the β_2-ARs in human airway epithelial cells and to determine the actions of the inverse agonist carvedilol on anion secretion in this cell system. Calu-3 cells were grown to confluence on membrane filters under ALI or LLI conditions until monolayers reached a transepithelial resistance of ~1000 $\Omega \cdot cm^2$ and were then mounted in Ussing chambers. Under ALI conditions, basolateral stimulation with epinephrine (1 μM) produced a large increase in CFTR-dependent short circuit current (Isc), consistent with stimulation of anion secretion that was significantly inhibited by the pan-specific β-AR antagonist propranolol. In contrast when cells were grown under LLI conditions, basolateral stimulation with 1 μM epinephrine produced a much smaller increase in Isc, indicating that culturing cells under ALI promotes the basoalteral expression of β-ARs. Apical stimulation with the β_2-AR agonist salbutamol (10μM) caused an increase in anion secretion similar to what was observed with 10μM 8cpt-cAMP. Initial treatment of Calu-3 cells with carvedilol caused a decrease in basal Isc and blocked the effect of 10μM 8cpt-cAMP on Isc. Pretreatment with the microtubule disrupting agent nocodazole blocked the inhibitory effects of carvedilol on Isc. Furthermore, western blot analysis of Calu-3 cells treated with carvedilol showed reduced expression of CFTR in the apical membrane, but no change in expression following stimulation with epinephrine.

These results suggest that carvedilol produces inhibition of cAMP-stimulated anion secretion by inducing endocytosis of CFTR from the apical membrane.

B. Introduction

Individuals with asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) use β-adrenergic receptor agonists to control and reduce symptoms of these respiratory diseases. These medications include short acting β-agonists, albuterol and long acting β-agonists, salmeterol (Patel et al. 2013; Ryall, Sillence, Lynch 2006). Each of these medications have selectivity for β_2-adrenergic receptors and are administered by inhalation. β-agonists act on airway smooth muscle to promote bronchodialation and increased airflow into the lung. Furthermore they stimulate mucociliary clearance by increasing ciliary beat frequency and epithelial fluid secretion (Bennett 2002; Benovic 2002; Wanner, Salathe, O'Riordan 1996). Mucociliary clearance is a defense mechanism that consists of a layer of mucus that rests on the surface of a periciliary liquid (PCL) layer that bathes the apical membrane and cilia of the airway epithelium. Inhaled pathogens and other foreign materials become trapped within the mucus layer and are subsequently transported out of the airways by directional ciliary movement toward the mouth (Ganesan, Comstock, Sajjan 2013; Kilburn 1968)

The airway surface epithelium is important in regulation of the PCL layer through the coordinated actions of ion channels and transporters that participate in transepithelial Na^+ absorption and anion secretion. The surface cells absorb sodium by a mechanism involving apical ENaC sodium channels and basolateral Na-K ATPase enzymes (Donaldson and Boucher 2007). Charge compensation and control of intracellular [K^+] is achieved by parallel movement of K^+ through K

channels present in the plasma membrane (Zhao et al. 2012). Inhibition of ENaC activity, either by addition of amiloride analogs or by reduced proteolytic activation of ENaC channels results in stimulation of anion secretion as a consequence of apical membrane hyperpolarization favoring anion efflux (Korbmacher et al. 2014; O'Grady, Jiang, Ingbar 2000). Secretion of chloride and bicarbonate induced by elevation of intracellular cAMP or $[Ca^{2+}]_i$ involves activation of apical CFTR or calcium activated Cl channels (TMEM16A) expressed in series with Na-K-2Cl cotransport (NKCC1) proteins and Na-K ATPase enzymes located in the basolateral membrane (Hollenhorst, Richter, Fronius 2011). Additionally, basolateral anion uptake can be mediated by Cl-HCO_3 excahnge or by Na-$(HCO_3)_x$ cotransport. Potassium recycling across the basolateral membrane can occur by cAMP-dependent activation of Kv7.1/MiRP2 channels or K_{Ca} channel opening in response to increases in $[Ca^{2+}]_i$ depending upon the origin of the cells. (Grahammer et al. 2001; Wang et al. 2008). Regulation of the depth of the PCL layer has been shown to be dependent on local autocrine signals including adenosine and nucleotide phosphates released from the airway epithelium and SPLUNC1, which controls proteolytic activation of ENaC in the apical membrane (Garcia-Caballero et al. 2009; Shan et al. 2011). Furthermore, changes in the thickness of the PCL layer are sensed and dynamically regulated by the surface epithelium in response to pathologic states such as microbial infection and allergic inflammation.

The production and secretion of mucus significantly contributes to mucociliary clearance. Submucosal glands are responsible for a large proportion

of mucus secretion in the upper airways (Ganesan, Comstock, Sajjan 2013). Delivery of mucus into the airway lumen is dependent on the coordinated regulation of serous and mucus cell secretions within the glands (Cho et al. 2012; Wine and Joo 2004) . Serous cells express high levels of CFTR in the apical membrane which facilitates anion secretion. Anion transport establishes an osmotic driving force for fluid secretion that increases the hydration of mucins secreted by mucus cells. (Wine and Joo 2004). Submucosal gland secretion is stimulated by cholinergic agonists that increase intracellular $[Ca^{2+}]_i$ and by β_2-adrenergic receptor ligands that increase cAMP-dependent phosphorylation of CFTR (Bennett 2002; Benovic 2002; Verkman, Song, Thiagarajah 2003; Wanner, Salathe, O'Riordan 1996; Wine and Joo 2004). In patients with obstructive airway diseases such as asthma, COPD or CF, mucociliary clearance is significantly impaired due to lack of normal CFTR function, enhanced ENaC activity and over production of mucus resulting from chronic airway inflammation (Ganesan, Comstock, Sajjan 2013; Knowles and Boucher 2002; Mall et al. 1996; Stutts et al. 1995; Wanner, Salathe, O'Riordan 1996). Reduced CFTR function and enhanced ENaC activity associated with the airway surface cells diminishes the depth of the PCL layer, resulting in compression of cilia by the mucus layer and restriction of ciliary movement. The increase in mucus production caused by activation of muscarinic receptor stimulation produces changes in PCL composition that can lead to formation of mucus plugs within smaller airways and increased risk of

infection (Knowles and Boucher 2002; Voynow and Rubin 2009; Wanner, Salathe, O'Riordan 1996).

In a recent study, epinephrine was reported to increase cAMP and intracellular Ca^{2+} in Calu-3 cells (an adenocarcinoma cell line expressing submucosal gland characteristics) grown under air-liquid interface conditions (Banga et al. 2014). This resulted in activation of CFTR and a Ca^{2+} activated chloride channels (CaCC) which resulted in an increase in transepithelial anion secretion. The initial transient increase in Isc was followed by a sustained current with diminishing oscillations over time. It was concluded that the CaCC responsible for the calcium-dependent component of the Isc was TMEM16A located in the apical membrane and together with CFTR these channels were responsible for the large increase in Isc produced by epinephrine and the associated oscillations. Moreover the results of this study indicated that TMEM16A and CFTR were functionally linked and coordinately regulated by increases in cAMP and $[Ca^{2+}]_i$.

The initial objective of the present study was to further examine the proposed interaction between TMEM16A and CFTR in Calu-3 cells using a CFTR deficient cell line. The second objective was to investigate the differential effects of the inverse β_2-AR ligand carvedilol on basal anion secretion and 8-cpt cAMP-evoked anion secretion. The results indicated that CFTR silencing abolished the Isc response to basolaterally applied epinephrine and that the inhibitory effects of carvedilol on basal and 8-cpt cAMP-induced anion secretion

were related in part to a down-regulation of CFTR protein expression in the apical membrane.

C. Materials and Methods

Materials

Eagles minimum essential medium with Earl's salts (MEM), fetal bovine serum (FBS), nonessential amino acids, penicillin-strepomycin and Phosphate-Buffered Saline (PBS) were purchased from Life Technologies (Carlsbad,CA). Trypsin was ordered from Lonza (Allendale NJ). Western blot protein markers, Magic Mark, See Blue, nitrocellulose membranes, 10% Bis-Tris gels and Enhanced Chemiluminescence (ECL) reagent were purchased from Life Technologies (Carlsbad, CA). Restore Western Blot Stripping Buffer and Cell Surface Protein Isolation kits were purchased from Thermo Scientific (Rockford, IL). Bovine Serum Albumin (BSA), carvedilol, epinephrine, 8cpt-cAMP, salbutamol and nocodazole were acquired from Sigma Aldrich (St Louis, MO). The selective β_2 receptor antagonist ICI-118,551 was obtained from Tocris Bioscience, (Bristol, UK). CFTR antibody (#2269) used for western blots was purchased from Cell Signaling (Danvers MA). β-tubulin primary antibodies and secondary goat anti-rabbit and goat anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti α-Tubulin for westerns was ordered from Millipore. Antibodies used for IHC, β_2-Adrenergic receptor antibody (Ab13989), anti-CFTR (Ab2784) antibody and secondary goat anti-chicken IgY Alexafluor

(Ab150169) antibodies were purchased from Abcam (Cambridge, MA). DAPI and slow fade reagent were purchased from Life Technologies (Carlsbad, CA).

Cell Culture

Human airway adenocarcinoma (Calu-3) cells were maintained in MEM medium containing 10% FBS, 1% nonessential amino acids and 1% penicillin-strepomycin in a humidified 5% CO_2 incubator at 37°C.

Apical membrane biotinylation

Calu-3 cells were grown on T75 flasks until 90% confluent. Cells were treated and membrane proteins isolated using a cell surface protein isolation kit from Thermo Scientific (Rockford, IL). Briefly, the procedure involved treating cells with 10μM epinephrine or carvedilol for 30 minutes. Cells were then washed in ice cold PBS and surface proteins labeled with biotinylation reagent (Sulfo-NHS-SS-Biotin). Labeled protein was subsequently harvested and affinity purified using NeutrAvidin Agarose Resin. Proteins were eluted using 50mM DTT in RIPA buffer and placed on a heat block for 5 minutes at 95°C, then spun at 1000 x g for 2 minutes. Isolated cell surface protein was then analyzed by western blot.

Western Blot analysis

Biotinylated membrane proteins were loaded onto a 10% bis-tris gel with a protein marker lane containing See Blue and Magic Mark. The gel was

transferred onto a nitrocellulose membrane and blocked in 5% milk/Tris-Buffered Saline with 0.2% Tween 20 (TBST) for 20 hours at 4°C. Membranes were then incubated overnight at 4°C with CFTR antibody (#2269) at a 1:1000 dilution and washed with TBST 3 times (5 minutes each wash), then incubated at room temperature for 1 hour with goat anti-rabbit IgG-HRP (1:7500 dilution). Membranes were then washed 3 times (5 minutes each wash) before incubation in ECL reagent for 1 min. After X-ray film exposure, blots were stripped for 5 minutes in Restore Stripping reagentand re-blocked in 5% milk/TBST overnight at 4°C. Membranes were then incubated in β-tubulin antibody (1:500 dilution) overnight at 4°C and washed 3 times in TBST before incubation with secondary goat anti-rabbit IgG-HRP antibody for 1 hour at room temperature. Afterwards, blots were placed in ECL reagent for 1 minute and exposed to X-ray film.

Immunohistochemistry

Cells were grown on Snapwell filters until confluent monolayers were formed. Monolayers were then fixed with 4% paraformaldehyde for 30 minutes followed by a 20 minute incubation with 0.1% TritonX100. Monolayers were washed 3 times between steps and blocked in 3% bovine serum albumin (BSA) for 2 hours, then incubated with primary β_2-AR antibody (Ab13989) and primary anti-CFTR antibody (Ab2784) overnight in 3% BSA. Monolayers were subsequently incubated in secondary Alexafluor antibodies diluted in PBS for 45 minutes and the filters washed 5 times with PBS. DAPI diluted in PBS was applied to the monolayers for a period of 10 minutes then removed by washing 5

times with PBS. Filters were then mounted on slides and membrane localization of β_2-AR and CFTR was determined by confocal microscopy (60X oil immersion).

Electrophysiology

Transepithelial resistances were measured using a EVOM epithelial voltohmeter attached to Ag/AgCl 'chopstick' electrodes [World Precision Instruments (WPI), New Haven, CT]. CFTR activity was measured using monolayers (~1000 $\Omega \cdot cm^2$) that were mounted in Ussing chambers and 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, which was maintained at 37°C and bubbled with 95% O_2-5% CO_2. CFTR was activated using 10μM 8cpt-cAMP and the short circuit current (Isc) was acquired using Axoscope8.0 software (Molecular Devices, Sunnvale CA)).

For experiments involving measurement of the apical membrane CFTR current, amphotericin B (15 μM) was used to perforate the basolateral membrane of the monolayers mounted in Ussing chambers. The basolateral (intracellular) surface was bathed with intracellular solution (in mM) 130 K-methanesulfonate, 5 KCl, 8 NaCl, 20 $KHCO_3$, 30 mannitol, 1 $MgSO_4$, 1 $CaCl_2$, and 10 glucose (pH 7.4), while the apical membrane (extracellular) was bathed with physiological saling solution (in mM) 70 Na-methanesulfonate, 5 KCl, 68 NaCl, 20 $NaHCO_3$, 1 $MgSO_4$, 1 $CaCl_2$, and 10 glucose (pH 7.4). CFTR was activated using 5 μM 8cpt-cAMP then subsequently treated with 10μM carvedilol, then 20 μM $CFTR_{inh}$-172

and the apical membrane currents acquired using pCLAMP 8.2 (Molecular Devices).

Statistical Analysis

Statistical significance was determined using an unpaired, two-tailed t-test (for single comparisons) or an ANOVA followed by a Bonferroni post test (for multiple comparisons). Results are expressed as the mean +/- standard error. A value of $p < 0.05$ was considered significant.

D. Results

Basolateral treatment of Calu-3 cells with 1μM epinephrine increased Isc in monolayers that were grown under ALI and LLI conditions (Figure 1). For both growth conditions the Isc increase was nearly abolished following treatment with the CFTR inhibitor, $CFTR_{inh}$-172. Additionally, the response toepinephrine was significantly greater for the ALI condition compared to the LLI condition. Pretreatment of cells with 10μM propranolol on the basolateral membrane effectively blocked the epinephrine response seen in control cells. Furthermore, epinephrine had no effect on Isc in shCFTR cells, indicating that the increase in Isc produced by β-AR activation was dependent on CFTR expression in the apical membrane.

Localization of CFTR and β2-AR were observed by immunohistochemistry using confocal microscopy. Calu-3 cells were grown on filters until confluent and fixed with 4% paraformaldehyde before exposure to CFTR and β_2-AR antibodies.

Fixed cells showed expression of both CFTR (in green) and the β2-AR (in red). Moreover, the yellow punctate labeling pattern indicates that the two proteins exhibit co-localization within the apical membrane of Calu-3 cells (Figure 2A). Figure 2B shows the effects of β_2-AR stimulation with salbutamol, a selective β_2-AR agonist and the effects of the cell permeable cyclic AMP analog, 8-cpt cAMP on Isc. The increase in current produced by apical salbutamol and 8cpt-cAMP reflects stimulation of transepithelial anion secretion. Addition of $CFTR_{inh}$-172 caused nearly complete inhibition of salbutamol evoked anion secretion. Furthermore apical addition of 10μM salbutamol to Calu-3 cells where CFTR expression was silenced by constitutive expression of shRNA (shCFTR cells) showed no increasae in anion secretion. These results demonstrate that CFTR is involved in producing the increase in Isc following stimulation with β_2-AR agonists. Similarly, monolayers treated with 10μM 8cpt-cAMP showed an increase in Isc that was absent in shCFTR cells. Figure 2C shows the time course of the 8cpt-cAMP effect on Isc. At the peak of the response, monolayers were apically treated with 10μM carvedilol, an inverse ligand that was previously shown to interact with the β_2-AR (Kveiborg et al. 2007; Vanderhoff, Ruppel, Amsterdam 1998). Unlike salbutamol, carvedilol produces a sustained decrese in Isc. However pretreating monolayers with 10μM ICI-118,551 (a selective β_2-AR antagonist) blocks the effects of carvedilol on 8cpt-cAMP evoked anion secretion (Figure 2D). Inhibition of the carvedilol response by ICI-115,551 demonstrates

that the effect of carvedilol is mediated by the β_2-AR. A summary of results from these experiments is presentedin figure 2E.

Data presented in figure 3 shows the effect of 10μM carvedilol on the basal Isc of Calu-3 cell monolayers. When the monolayers were subsequently treated with 10μM 8cpt-cAMP no significant effect on Isc was observed (Figure 3A). In contrast, monolayers pretreated with 10μM ICI-118,551 did not exhibit a decrease in basal Isc following apical addition of 10μM carvedilol. Furthermore, ICI-118,551 rescued the effect of 8cpt-cAMP on anion secretion (Figure 3B). These experiments demonstrate that treatment with carvedilol inhibits basal anion secretion and it is dependent on activation of the β_2-AR. A summary of these results is presented in Figure 3C.

To determine whether the effect of carvedilol on Isc was the result of CFTR inhibition, apical membrane Cl^- currents were measured in Calu-3 monolayers where the basolateral membrane resistance was eliminated following treatment with amphotericin B. For these experiments the basolateral surface of the monolayer was bathed in intracellular solution and the apical membrane was bathed in physiological saline solution. The apical membrane was held at zero mV and stepped from -30 to +30 mV in 5 mV increments to measure the CFTR conductance. CFTR was stimulated by treating the cells with 8cpt-cAMP, which evoked a significant increase in the slope of the $CFTR_{inh}$-172 sensitive current-voltage (I-V) relationship. Addition of 10μM carvedilol inhibited the 8cpt-cAMP evoked Cl^- current and significantly reduced the CFTR conductance (Figure 4A).

In contrast, no significant increase in apical Cl^- current or conductance was observed in shCFTR cells (Figure 4B). These results indicate that the inhibitory effect of carvedilol on 8cpt-cAMP stimulated anion secretion in Calu-3 cells is the result of a decrease in CFTR activity. To determine if the decrease in CFTR conductance was associated with a reduction in apical CFTR expression, Calu-3 cells were exposed to 30μM nocodazole for 2 hours to disrupt microtubule-dependent retrieval of proteins from the plasma membrane. Cells treated with nocodazole showed microtubule disruption as indicated by a decrease in tubulin-containing filaments (Figure 5A). Furthermore, western blot analysis revealed that cells treated with nocodazole for 30 minutes, 1 hour and 2 hours exhibited a decrease in monomeric α-tubulin associated with the NP-40 insoluble fraction, indicating an increase in microtubule depolymerization (Vossenkamper et al. 2007) (Figure 5B). The effect on CFTR conductance was then determined using monolayers pretreated with 30μM nocodazole prior to stimulation with 10μM 8cpt-cAMP and subsequent treatment with carvedilol. Cyclic AMP caused an increase in CFTR conductance in both control and treated cells, however addition of 10μM carvedilol failed to reduce CFTR conductance as observed in control monolayers (Figure 5C). These results suggest that a portion of the inhibitory effect of carvedilol on anion secretion is dependent on an intact microtubule network.

The effect of microtubule disruption on carvedilol mediated inhibition of anion secretion is shown in Figure 6. Monolayers were initially treated with 30μM

nocodazole for 2 hours prior to stimulation with 10μM 8cpt-cAMP. Nocodazole treated monolayers exhibited an increase in Isc that was similar to control conditions. However the effect of carvedilol after addition of 8cpt-cAMP was blocked in nocodazole treated cells (figure 6C). These results are consistent with the data reported in figure 5 demonstrating that carvedilol inhibition of Isc was dependent on a functional microtubule network.

Data presented in figure 7 shows the level of CFTR protein expression within the apical membrane of Calu-3 cells using surface biotinylation. For these experiments cells were treated with 10μM carvedilol or epinephrine prior to biotinylation. Carvedilol treatment showed a significant decrease in CFTR protein expression in the apical membrane compared to the control cells. In contrast, epinephrine showed no significant decrease in apical expression compared to the controls (Figure 7C). These results suggest that at least a portion of the inhibitory actions of carvedilol on 8ctp-cAMP stimulated anion secretion results from a decrease in CFTR protein levels at the plasma membrane.

E. Discussion

An interesting and previously unreported finding from the present study was the observation that the response to β-AR agonist stimulation on the basolateral membrane depends on growth conditions. Cells grown under ALI produced a significantly greater response to basolateral stimulation with epinephrine compared to cells under LLI conditions. One possible explanation for

this effect is that the expression of β-AR protein in the basolateral membrane is enhanced under ALI resulting in a larger Isc response when stimulated with β-AR agonist. Another possibility is that coupling between the β-AR and adenylyl cyclase is enhanced, resulting in a greater level of cAMP production relative to the LLI condition. It is also possible that the larger Isc response is not directly related to β-AR expression or signaling, but is the result of a change in the level of K_v7.1/MiRP2 expression in the basolateral membrane, which could lead to a larger increase in basolateal K^+ efflux and a greater driving force for Cl^- exit acrosss the apical membrane. Additional experiments will be necessary in order to determine whether one or more of these possibilities is responsible for the effects of ALI on anion secretion.

β_2-AR are G-Protein coupled receptors (GPCR) that when stimulated with an agonist such as salbutamol, activation of G_s signaling occurs. This leads to downstream activation of adenylyl cyclase and cAMP (Benovic 2002) When an agonist binds to a receptor causing activation of the receptor (Leurs, Church, Taglialatela 2002). Inverse agonists bind to the inactive state of a receptor and promote an opposite response of an agonist, eliminating basal activity of the receptor (DG Lambert 2004; Leurs, Church, Taglialatela 2002; Maack et al. 2000; Maack et al. 2001; Parra and Bond 2007). Biased ligands bind to a receptor and elicit a selective signal while eliminating and or preventing other signals produced by the receptor (Steen et al. 2014; Tao and Conn 2014). Carvedilol is an inverse agonist commonly used in cardiovascular medicine for heart failure and

hypertension whose effect include increase of left ventricular function, and reductions of blood pressure (Vanderhoff, Ruppel, Amsterdam 1998). It does so by binding to β-adrenergic receptors leading to vasodilatation and decreasing heart rate (Kveiborg et al. 2007). Little is known about the effects of carvedilol on airway epithelial cells. These studies show that treating cells with carvedilol after stimulation with 8cpt-cAMP, a known activator of CFTR dependent anion secretion in airway epithelial cells (Schultz et al. 1999) causes a significant decrease in activated anion secretion as well as basal current decrease carvedilol pretreatment. Measurements of the apical membrane anion conductance following stimulation with 8cpt-cAMP revealed that the carvedilol-induced decrease in Isc resulted from the inhibition of CFTR. These results are consistent with carvedilol acting as an inverse agonist. The ability of ICI-118-551 to block the carvedilol effect on anion secretion and to rescue the 8cpt-cAMP response suggests that the effect of carvedilol is mediated through the β_2-AR and does not act as a CFTR blocker.

To determine if carvedilol-induced inhibition of CFTR activity was due to internalization of the channel, nocodazole was used to depolymerize microtubules and decrease vesicle transport (Vasquez et al. 1997; Vossenkamper et al. 2007). Treatment of Calu-3 cells for 30 minutes with nocodazole produced a significant reduction in alpha-tubulin polymerization and rescued CFTR channel activity and 8cpt-cAMP dependent anion secretion in carvedilol treated cells. These results provide indirect evidence suggesting that

the effect of carvedilol is due to removal of CFTR channels from the apical membrane. Furthermore, subsequent apical surface biotinylation experiments revealed that carvedilol stimulation resulted in a significant decrease in CFTR protein expression compared to untreated controls and epinephrine treated cells. These results indicate that carvedilol inhibits CFTR channel function by stimulating internalization of the channel.

It has been shown in previous studies that β_2-AR and CFTR co-localize in airway epithelia forming a signaling complex at the apical membrane (Naren et al. 2003; Taouil et al. 2003). This signaling complex includes NHERF and EBP50 which physically couple β_2-AR and CFTR through interactions with their respective PDZ domains. These scaffolding proteins help to stabilize CFTR at the apical membrane (Taouil et al. 2003). When an agonist such as epinephrine binds to the β_2-AR receptor two distinct signaling pathways are activated. Release of G_s α subunit from the β_2-AR ultimately increases CFTR channel activity by increasing production of cAMP and stimulation of PKA-mediated phosphorylation of the channel. (Naren et al. 2003; Taouil et al. 2003) . Activation of PKA and phosphorylation of CFTR is thought to be responsible for the disassociation of the CFTR and β_2-AR complex (Naren et al. 2003). The second pathway activated by binding of epinephrine involves β-arrestin-dependent signaling. After a period of time following agonist binding to the receptor G-Protein receptor kinases (GRKs) are recruited to the plasma membrane where they phosphorylate the C-terminal tail of the β_2-AR (Benovic 2002; Han et al.

2012; Reiter et al. 2012). Conformational changes associated with this phosphorylation facilitate binding of β-arrestin, a cytosolic adaptor protein, to the receptor (Shenoy et al. 2009; Shenoy and Lefkowitz 2011). This allows for recruitment of Nedd4 an E3 ligase that ubiquinates the receptor effectively labeling the protein for internalization and targeting the receptor to lysosomes (Shenoy et al. 2007; Shenoy 2007; Shenoy et al. 2008; Shenoy and Lefkowitz 2011). However when carvedilol binds to the β_2-AR, a distinct conformational change occurs that blocks G_s signaling, but allows for GRK recruitment (Kim et al. 2008; Kim et al. 2005; Shenoy and Lefkowitz 2011; Wisler et al. 2007). The resulting β-arrestin binding recruits MARCH2, a different E3 ligase that ubiquinates the C-terminal tail of the receptor (Han et al. 2012). This leads to protein internalization and degradation through a process of endocytosis and lysosomal degradation (Han et al. 2012; Shenoy and Lefkowitz 2011; Wisler et al. 2007). Unlike epinephrine where the β_2-AR-CFTR signaling complex disassociates after epinephrine binding leading to β_2-AR internalization and leaving CFTR in the apical membrane, carvedilol stimulation may not allow for the β_2-AR-CFTR complex to disassociate. This is due to the inhibition of G_s signaling (Han et al. 2012; Naren et al. 2003). Presumably, this would result in internalization of the entire signaling complex.

There have been many studies on the therapeutic potential of bias ligands in a variety of diseases including cardiovascular, metabolic, osteoporosis, HIV and cancer. For example carvedilol is used in treatment of cardiovascular

disease because it provides cardioprotection by promoting increased expression of epidermal growth factor receptor (EGFR) and preventing the activation of G-Protein signaling, which when chronically stimulated may have detrimental cardiac effects (Drake et al. 2008; Wisler et al. 2007). The angiotension II type 1 receptor (AT1R) is another receptor that β-arrestin bias ligand stimulation may provide beneficial effects to patients with heart failure by increasing cardiac function (Whalen, Rajagopal, Lefkowitz 2011a; Whalen, Rajagopal, Lefkowitz 2011b). The effects of β-arrestin bias ligands on patients with osteoporosis has been shown to have great potential by increasing production of anabolic bone formation without activating bone resorption. G-Protein bias ligands also have great potential for treating diseases such as congenital nephrogenic diabetes insipidus (cNDI) a disease that has a mutated vasopressin receptor (V2R). In cND1 G-Protein inverse ligands act as a pharmacologic chaperones aiding in the restoration of the V2R misfolded protein, rescuing receptor function and promoting G-Protein signaling while preventing β-arrestin signaling (Whalen, Rajagopal, Lefkowitz 2011a). The prevention of β-arrestin signaling prevents the internalization of the receptor allowing the receptor to be available in the membrane for continuous stimulation.

It has also been suggested that inverse agonists may play an important therapeutic role in diseases that involve constitutively active GPCR because of their effects on decreasing the basal activity of the receptor (Tao and Conn 2014). Previous studies have shown the potential therapeutic use of β-inverse

agonists on asthma in the murine model by decreasing inflammatory cells and cytokines (Matera, Calzetta, Cazzola 2013; Nguyen et al. 2008). Inverse agonists have also been thought to have therapeutic benefits in metabolic disease such as obesity (Cameron, Bhattacharya, Loomis 2014; Tao and Conn 2014) playing a role as pharmacologic chaperones, correcting misfolded proteins (Tao and Conn 2014) and acting as GPCR blockers, limiting the constitutive activity of the receptors (Cameron, Bhattacharya, Loomis 2014). Studies on the use of bias ligands and inverse agonists as therapeutic agents will continue to be done focusing on the reduction of potential detrimental side effects and promoting symptom relief as well as potential disease reversal.

It is evident through these studies that targeting receptors with inverse agonists not only help in the understanding of downstream signaling mechanism(s) of the β_2-AR-CFTR signaling complex but also could potentially serve as a tool to develop new drugs for chronic β-agonist users. These new drugs could work in the opposite way of carvedilol, targeting the G_s signaling pathway and promoting CFTR activity while limiting the negative effects of GRK signaling.

Figure 1

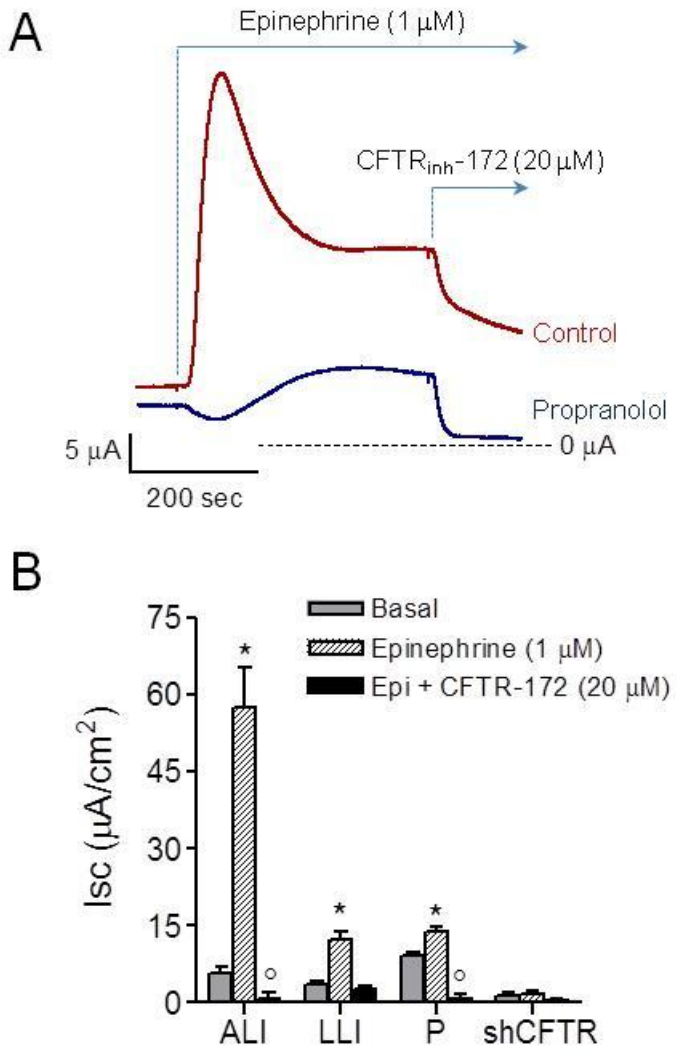

Figure 1: Epinephrine stimulated anion secretion on basolateral membrane of Calu-3 cells where propranolol blocked anion secretion. **A:** Isc tracing showing the effects of epinephrine and propranolol (10μM) on the evoked anion secretion of basolateral membranes of Calu-3 cells grown in air liquid interface (ALI). **B:** Summary bar graph showing the effects of epinephrine (1μM) and epinephrine + $CFTR_{inh}172$ (20 μM) on basolateral membrane Isc of wild type Calu-3 cells in ALI, wild type Calu-3 cells in liquid liquid interface (LLI), propranolol treated (P) Calu-3 cells, or CFTR deficient (shCFTR) Calu-3 cells.

Figure 2

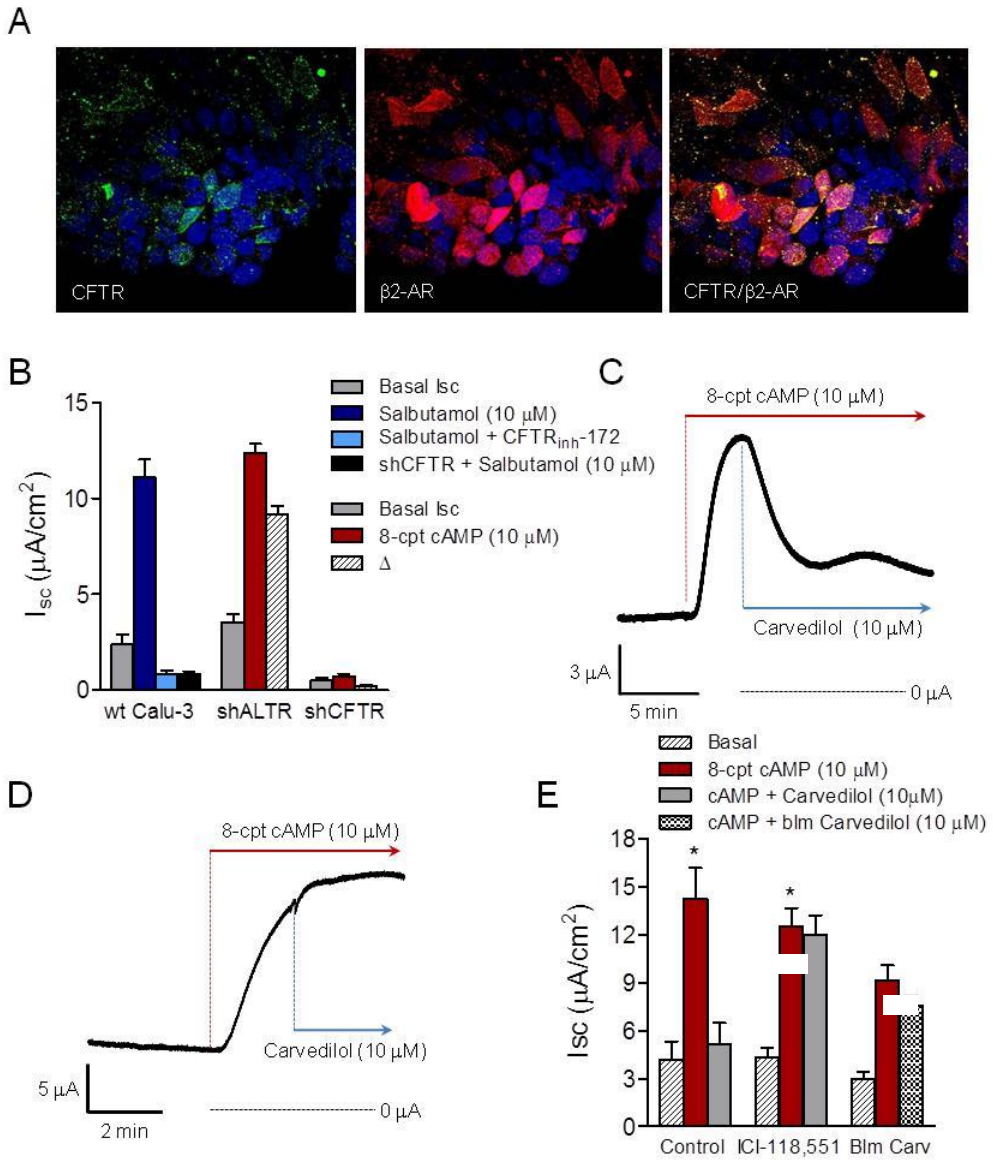

Figure 2: Immunohistochemistry and effects of β_2-AR ligands on 8cpt-cAMP (10μM) stimulated anion secretion. **A:** Confocal images showing membrane localization of CFTR (green), β_2-AR (red) and the superimposed image of both antibody-labeled proteins expressed in Calu-3 cells. The yellow color indicates co-localization of the CFTR and β_2-AR in the superimposed image. **B:** Summary bar graph showing the effects of salbutamol (10μM) and 8cpt-cAMP (10μM) on Isc of wild type Calu-3 cells or CFTR deficient (shCFTR) Calu-3 cells. Resulst show the change in Isc (Δ = hatched bar) after salbutamol or 8cpt-cAMP treatment from basal Isc. **C**: Isc tracing showing the effects of carvedilol on 8-cpt cAMP evoked anion secretion. **D**: Isc tracing showing that the β_2-AR-selective antagonist ICI-118,551 (10μM) blocks the inhibitory effect of carvedilol on 8cpt-cAMP evoked anion secretion. **E**: A summary of Isc results comparing control monolayers to monolayers pretreated with ICI-118551 and monlayers treated on the basolateral membrane (BLM).

Figure 3

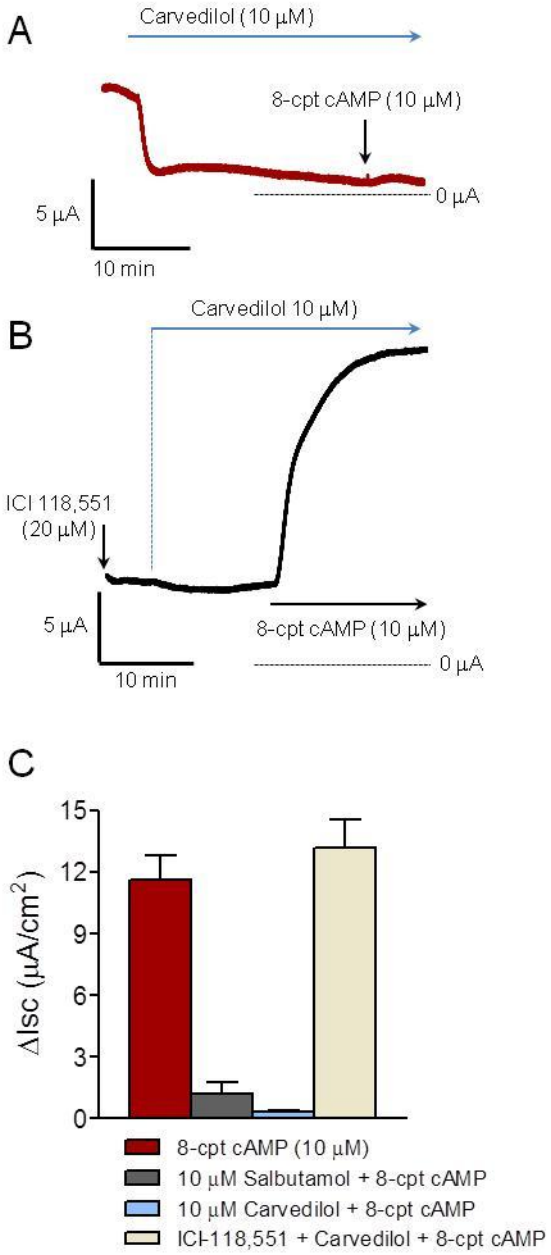

Figure 3: Carvedilol inhibits basal and 8cptcAMP stimulated anion secretion whereas the β_2-AR antagonist ICI-118,551 rescues 8cpt-cAMP evoked anion secretion. **A:**Carvedilol (10μM) decreases the basal current and blocks the effects of 8cpt-cAMP on anion secretion. **B:** Monolayers pretreated with 10μM ICI-118551 blocks the effect of carvedilol on basal anion secretion and rescues the effect of 8cpt-cAMP on Isc. **C:** Summary graph showing the effects of salbutamol, 8cpt-cAMP and carvedilol on Isc.

Figure 4

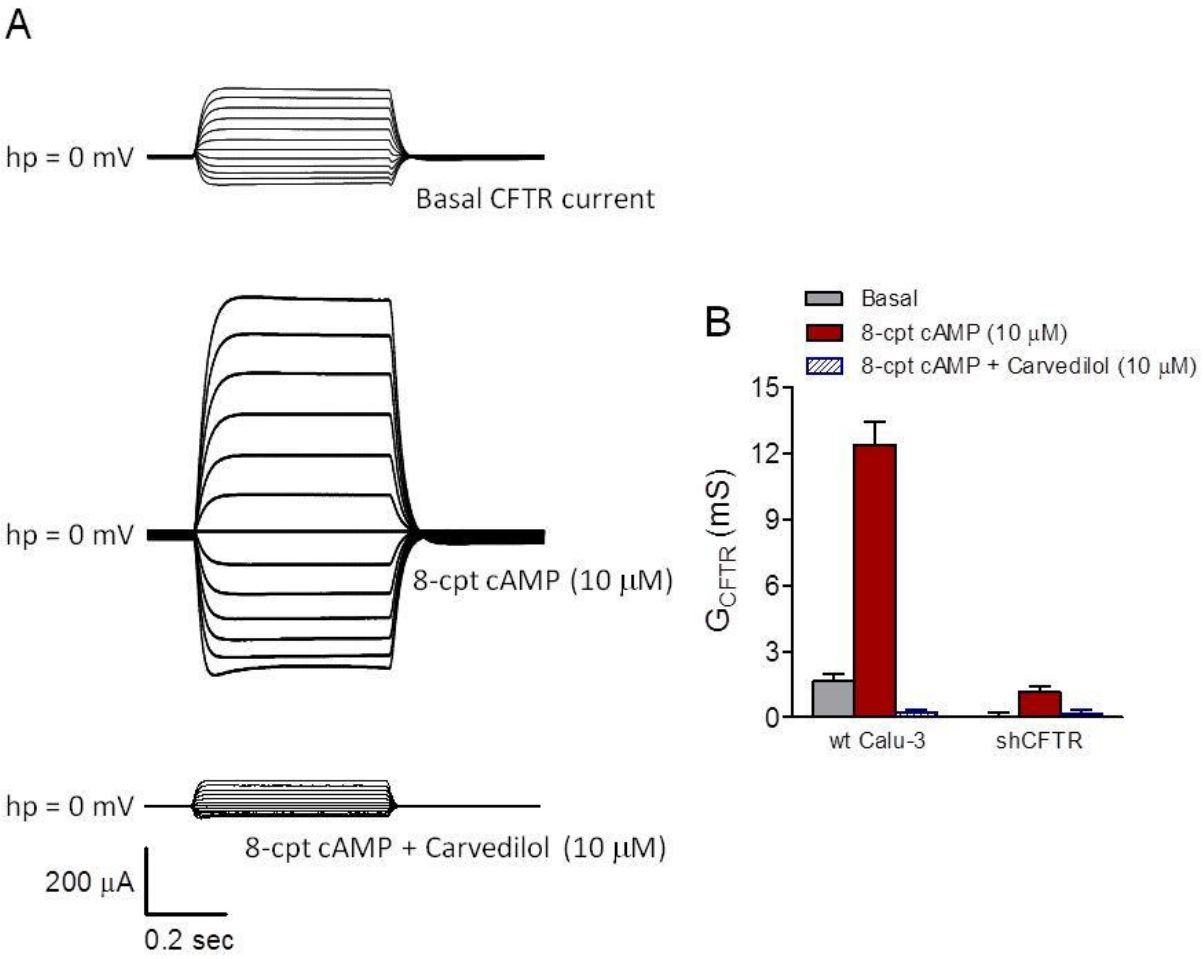

Figure 4: Measurement of $CFTR_{inh}$-172 sensitive apical membrane currents using amphotericin B permeablized Calu-3 monolayers. **A:** Basal, 8cpt-cAMP (10μM) stimulated and 8ctp-cAMP stimulated, carvedilol inhibited CFTR currents evoked by a voltage step protocol where monolayers were held at zero mV and stepped from -30 to +30 mV in 5 mV increments. **B:** Apical membrane conductance measurements obtained from the slopes of I-V relationships of experiments shown in part A. (apical current traces of wild type Calu-3 cells and shCFTR cells at rest (grey), after addition of 8cpt-cAMP (red) and following addition of carvedilol to the apical solution after stimulation with 8cpt-cAMP (blue hatched).

Figure 5

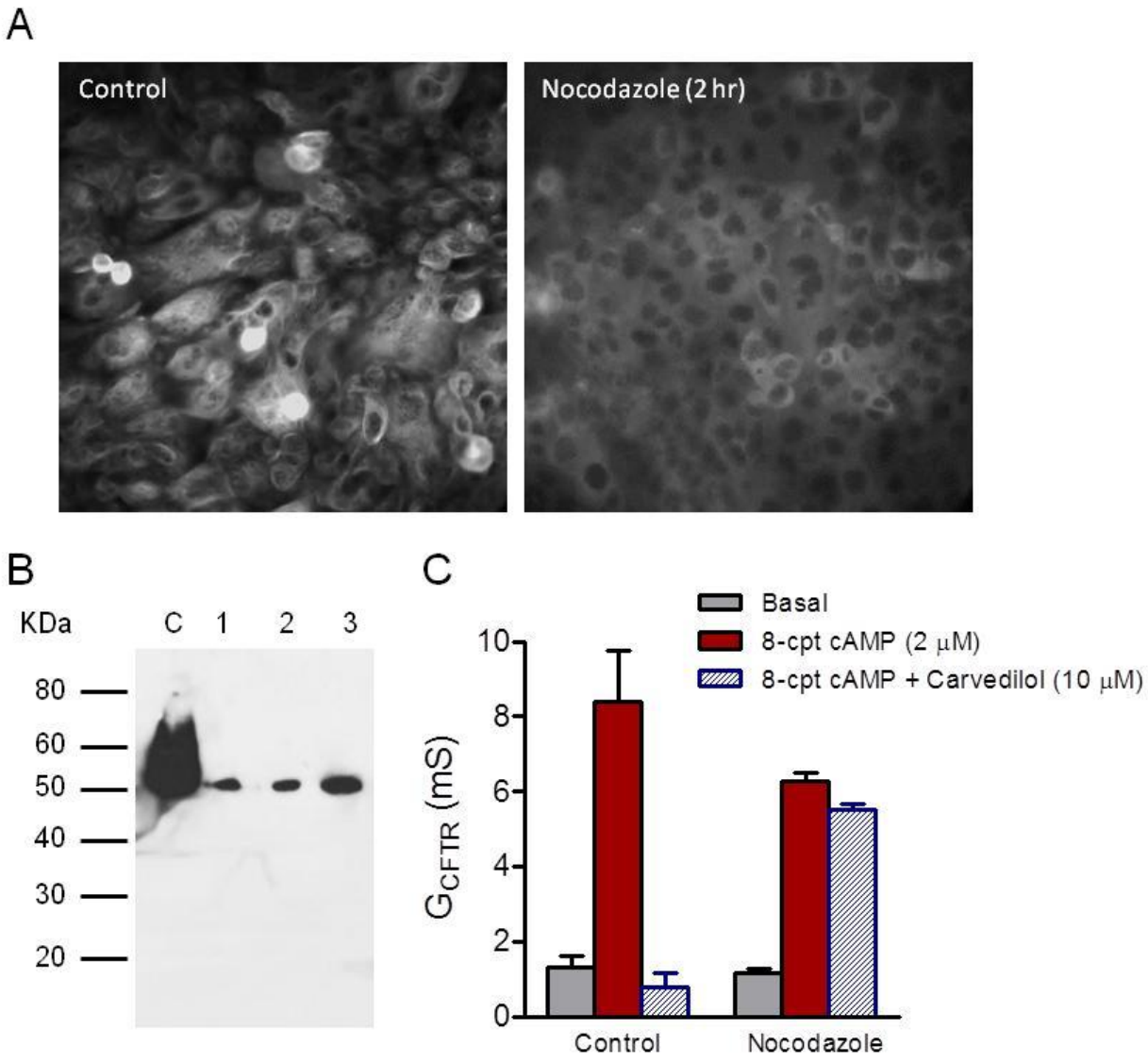

Figure 5: Effects of microtubule disruption with nocodazole on carvedilol-dependent inhibition of 8cpt-cAMP stimulated anion secretion. **A:** Images of Calu-3 cells 2 hours after incubation with 30 μM nocodazole; Note the loss of filament structure compared to untreated control cells. **B:** Western blot analysis of α-tubulin protein expression in Calu-3 cells treated with Nocodazole at 0 minutes (C,), 30 minutes (lane 3), 1 hour (lane 2) and 2 hours (lane 1) **C:** Bar graph displaying the effect of carvedilol mediated inhibition of CFTR conductance in control and nocodazole treated cells.

Figure 6

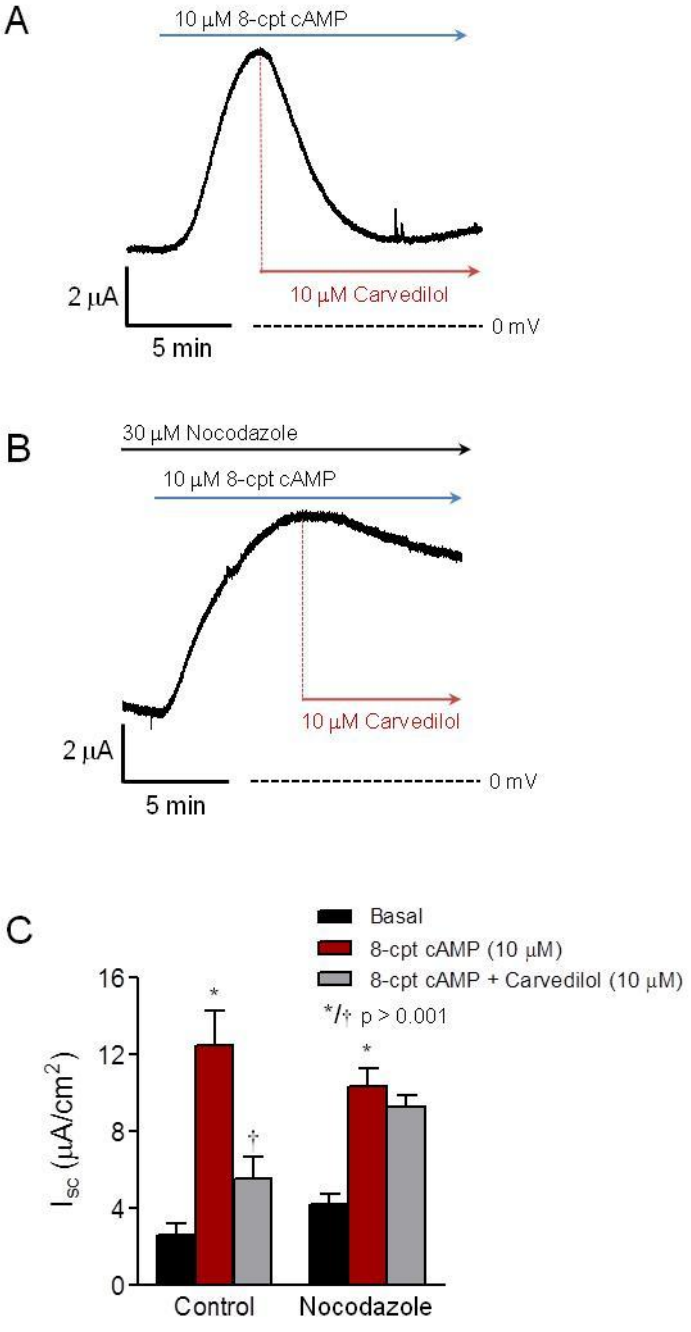

Figure 6: The effects of nocodazole on carvedilol-dependent regulation of 8cpt-cAMP evoked anion secretion. **A:** 8cpt-cAMP dependent anion secretion is inhibited by addition of 10μM carvedilol. **B:** Pretreatment of monolayers with 30μM nocodazole for 2 hours blocks the inhibitory effects of carvedilol on 8cpt-cAMP stimulated anion secretion. **C:** Summary graph showing that the effect of carvedilol on the 8cpt-cAMP stimulated increase in Isc was dependent on a functional microtubule network.

Figure 7

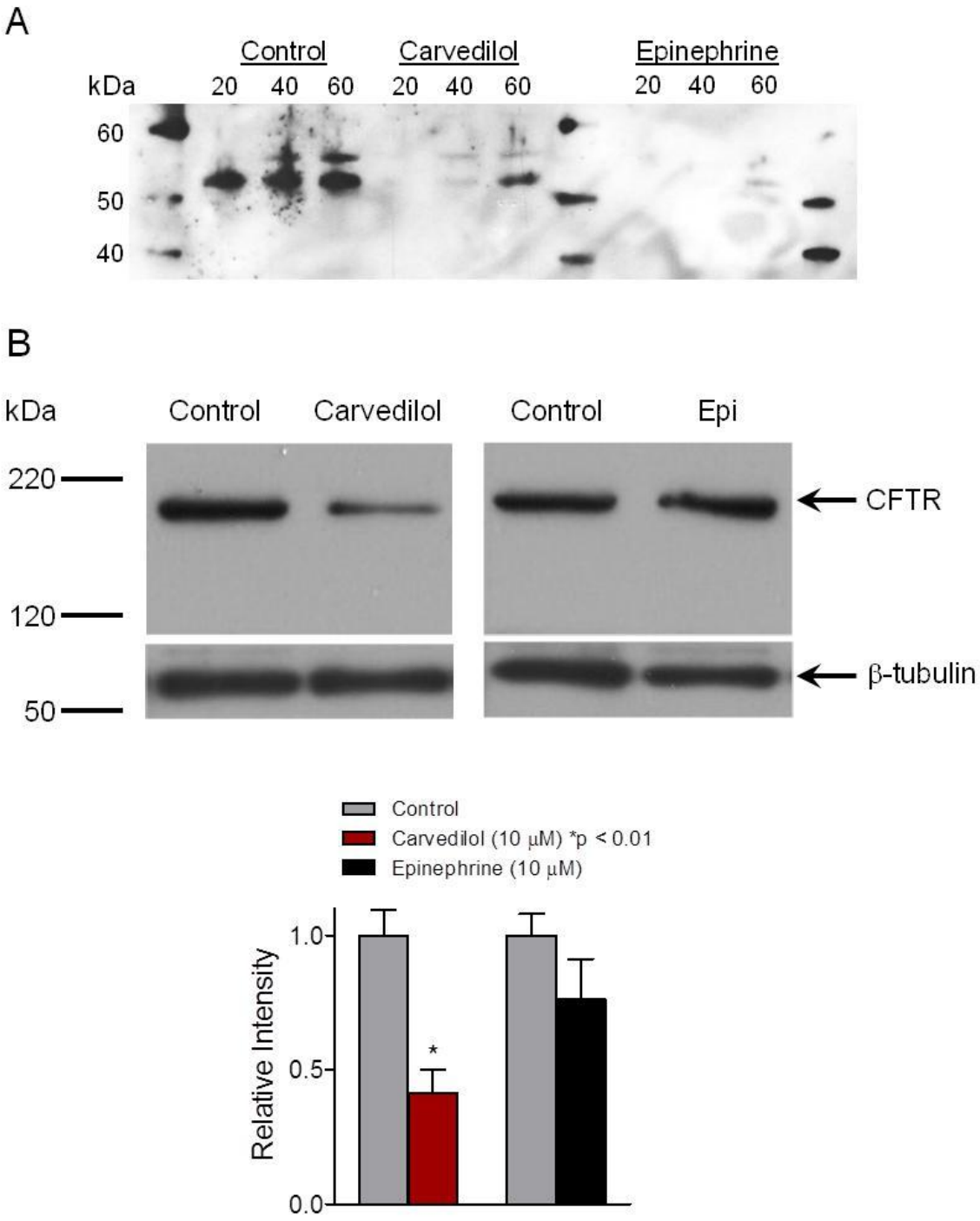

Figure 7: Western blot analysis showing the effects of carvedilol and epinephrine on expression of CFTR in the plasma membrane. **A:** Western blot showing CFTR and β-tubulin (loading control) protein present in biotinylated membranes from Calu-3 cells treated with carvedilol or epinephrine relative to untreated control cells. **B:** Analysis of the relative intensity of CFTR bands from four separate biotinylation experiments revealing a decrease in the level of CFTR protein following treatment with 10μM carvedilol for 30 minutes.

Chapter 3

β_2-Adrenergic Receptor Stimulation Inhibits Cell Migration and Epithelial Restitution by a Mechanism Involving CFTR

A. Summary

The objective of this study was to investigate the effects of β-adrenergic receptor stimulation on airway epithelial cell migration and its effects on CFTR activity in airway epithelial cells. These studies were performed using normal human bronchial epithelial (NHBE) cells and Calu-3 cells which were grown to confluence on impedance sensing chamber arrays, each containing a circular 250 μm diameter electrode. Wound repair was monitored by continuously applying alternating current (~1 μA, 15 kHz) and measuring increases in impedance as cells migrated onto the electrode surface. Exposure to 10μM epinephrine produced a significant delay in wound closure while pretreatment with the pan-specific β-adrenergic receptor antagonist propranolol (10μM) abolished the inhibitory effects of epinephrine. These results were also confirmed in human vaginal epithelial cells (hVE) and human cervical epithelial cells (hCE). The inhibitory effect on migration was also observed after stimulation with the selective β_2-AR agonist salbutamol (10μM) however no effect was observed when cells were treated with 10μM dobutamine a β_1-AR specific agonist. These results indicated that the effect on migration resulted from activation of the β_2-AR. Moreover, inhibition of CFTR activity by addition of 20μM $CFTR_{inh}$-172 or using CFTR knockout cells blocked any further decrease in migration rate after addition of 10μM epinephrine, signifying that the effects on cell migration were dependent on CFTR activity. Carvedilol, an inverse ligand, was also shown to cause a decrease in cell migration and wound repair similar to the effects of epinephrine.

Furthermore a reduction in lamellipodia area was observed after epinephrine treatment that was similar to the effect of $CFTR_{inh}$-172. These results demonstrated that airway epithelial migration and wound repair is significantly reduced following exposure to β-adrenergic receptor agonists and that this effect involves inhibition of CFTR channel activity.

B. Introduction

The conducting airways are composed of several cell types, including basal, secretory, ciliated and neuroendrocrine cells (Hackett et al. 2011; Hajj et al. 2007a; Roomans 2010; Yang and Chen 2014), which play an important role in maintaining a functional airway epithelium. When the epithelium is injured, regeneration and repair take place. Basal cells are progenitor cells within the airways that can differentiate into ciliated or secretory cells, (Crystal et al. 2008; Hajj et al. 2007a; Rock et al. 2009; Roomans 2010; Voynow et al. 2005; Yang and Chen 2014). There is also evidence that Clara cells can differentiate into ciliated cells following injury.(Hajj et al. 2007a; Hong et al. 2001; Stripp and Reynolds 2008; Teisanu et al. 2011; Voynow et al. 2005; Yang and Chen 2014) The process of wound repair typically involves the migration of progenitor cells into the damaged region of the epithelium. Cells at the edge of the wound form lamellipodia, pseudopod-like structures that facilitate ameoboid movement that allow the cells to migrate towards the center of the wound (Coraux et al. 2008; Lu, Reinach, Kao 2001). The migration is directed in part, by an electric field (EF) that spontaneously occurs with development of the wound (Chiang, Robinson, Vanable 1992; Soong et al. 1990; Sun et al. 2011; Zhao et al. 1996a). This EF has been shown to direct migrating cells by a process commonly referred to as electrotaxis (Song et al. 2002; Zhao et al. 1996a; Zhao et al. 1996b). A previous study by Schiller et al. (2010) showed that CFTR was essential in the migration of airway epithelial cells and Sun et al. (2011) showed that the channel was

responsible for the EF generated after wounding. Patients that suffer from Cystic Fibrosis (CF) lack functional CFTR in the apical membrane of airway epithelial cells and there is evidence indicating that these individuals are unable to effectively and efficiently repair damaged epithelium after injury, contributing to chronic infection and inflammation (Boucher 2004; Chmiel and Davis 2003; Guggino and Stanton 2006; O'Sullivan and Freedman 2009; Wanner, Salathe, O'Riordan 1996).

Most individuals that suffer from CF, asthma or COPD take β-adrenergic receptor (AR) agonists to dilate their airways to increase airflow into the lungs and to increase ciliary beat frequency aiding in mucociliary clearance (Cazzola and Matera 2014; Salathe 2002; Ulrik 2014). However it has been shown that β_2-AR agonists can lead to a loss in bronchioprotection, resulting in an increased risk of infection (Bhagat, Swystun, Cockcroft 1996; Booth et al. 1996; Ramage et al. 1994; Spina 2014). Long acting β_2-AR agonists such as salmetrolol have been shown to cause down-regulation of the β_2-AR (Walker et al. 2011; Wasilewski, Lougheed, Fisher 2014). This leads to a decrease in availability of the receptor to promote bronchiodilatation preventing fast acting, therapeutic effects of the agonist (Spina 2014; Walker et al. 2011; Wasilewski, Lougheed, Fisher 2014). Furthermore, β-agonists have been shown to decrease the rate of cell migration in other epithelial cell types including keratinocytes and corneal epithelial cells (Chen, Hoffman, Isseroff 2002; Pullar et al. 2006; Pullar et al. 2007). However less is known about their effects in airway epithelia.

The objective of the present study was to investigate the effects β-agonists have on airway epithelial cell migration during the process of wound closure using Calu-3 cells, a model airway epithelial cell line of submucosal gland origin and in normal human bronchial epithelial (NHBE) cells. The effects of β-agonists on vaginal and cervical epithelial cell migration were also investigated. Additionally, the effect of the inverse agonist carvedilol on migration was also determined using airway epithelial cells. The results demonstrated that β-agonists produced a significant inhibition of cell migration in each of these cell systems during wound repair, similar to what was previously observed in keratinocytes and corneal epithelial cells. Inhibition of CFTR blocked the effect of β-agonists on airway epithelial cell migration, indicating that loss of CFTR function was associated with the decrease in migration rate. Additionally, β-agonists caused diminished protrusion of lamellipodia similar to the actions of the CFTR inhibitor $CFTR_{inh}$-172. Our results of this study provide evidence that stimulating the β_2-AR with an agonist or inverse ligand leads to a decrease in CFTR function using two different mechanisms, both of which cause a significant decrease in the cell migration rates of these cells.

C. Materials and Methods

Materials

Eagles minimum essential medium with Earl's salts (MEM), fetal bovine serum (FBS), nonessential amino acids, penicillin-strepomycin and Phosphate-

Buffered Saline (PBS) were purchased from Life Technologies (Carlsbad,CA). Bronchial epithelial cell media BEGM with bullet kits and Trypsin were ordered from Lonza (Allendale NJ). Bovine Serum Albumin (BSA), $CFTR_{inh-}$ 172, carvedilol, epinephrine, 8cpt-cAMP, salbutamol, L-Cysteine were acquired from Sigma Aldrich (St Louis, MO). The selective β_2 receptor antagonist ICI-118,551 was obtained from Tocris Bioscience, (Bristol, UK). Bovine Collagen Type 1 was purchased from BD biosciences (San Jose, CA).Texas Red X phalloidin, SlowFade Gold antifade and Superscript II reverse transcriptase were purchased from Invitrogen. RNeasy Mini Kit was purchased from Qiagen. Turbo DNase was purchased from Ambion (Applied Biosystems Foster City, CA), SYBR green Brilliant III master mix and passive reference dye for qualitative real-time PCR (qRT-PCR) were purchased from Agilent Technologies (Santa Clara, CA). ECIS arrays were purchased from Applied Biophysics (Troy, NY).

Cell Culture

Normal Human Bronchial Epithelial (NHBE) cells were maintained in complete bronchial epithelial cell growth media (BEGM) with provided bullet kits which are SingleQuots of bovine pituitary extract, epinephrine, gentamicin/amphotericin-B, human epidermal growth factor, hydrocortisone, insulin, retinoic acid, transferrin, and triiodothyronine. The human airway adenocarcinoma cell line Calu-3 WT, shCFTR and shALTR cells were maintained in MEM medium containing 10% FBS, 1% nonessential amino acids and 1% penicillin-strepomycin. The human vaginal epithelial cells (hVE) and the

ectocervical cells hCE were obtained from ATCC (Manassas, VA) and were maintained in Keratinocyte-SFM (1X) from life technologies (Carlsbad,CA), containing 10% FBS and 0.1% penicillin-strepomycin. All cells were maintained in a humidified 5% CO_2 incubator at 37°C.

Quantitative RT-PCR

Expression of β-AR was determined by qRT-PCR. Total RNA was extracted from monolayers using RNeasy Mini Kit from Qiagen (Valencia, CA). Messenger RNA (mRNA) was DNase treated using TURBO DNase prior to generating complimentary DNA (cDNA) using reverse transcriptase (RT) following the manufacturer's protocol. Quantitative PCR reactions were done using Brilliant III SYBR green detection of newly synthesized PCR products following manufacturer's protocol. RT reactions were diluted 10 fold for each qRT-PCR reaction. Each qRT-PCR reaction had forward and reverse primers at a final concentration of 10μM. Primers used for the qRT-PCR reactions are shown in Table 1.

Electrical cell substrate impedance sensing (ECIS)

ECIS was used to measure NHBE and Calu3 cell migration. Cells were grown on 8W1E ECIS arrays, maintained in complete growth media until fully confluent. Media was then changed to serum-free for 48 hours. A 250 μm wound was produced by electroporation (6V, 30 kHz, 60 seconds) of cells in contact with the electrode. Cells were washed with PBS and treatment media was added.

Impedance was measured using an alternating current (~ 1μA, 15 kHz) that was continuously applied to the electrodes. Wound closure was measured for each treatment by determining the time required to reach 50% of the maximum impedance.

Lamellipodia Assay

A lamellipodia protrusion assay was performed using NHBE and Calu-3 cells under the following treatment conditions: 10μM epinephrine or 10μM salbutamol, 20μM $CFTR_{inh}$-172, 20μM $CFTR_{inh}$-172 + 10μM epinephrine and an untreated control condition. Chamber slides were coated with bovine collagen diluted in 0.01N acetic acid. Calu-3 cells were seeded on precoated slides and maintained using standard conditions and growth media until confluent. Cells were changed to SF media for 48 hours. Scratch wounds were made using a scalpel blade dipped in liquid N2; cells were washed in PBS and exposed to treatment conditions for 60 minutes. This treatment exposure time was previously shown to be sufficient for cells to produce lamellipodia and start the process of migration (Schiller, Maniak, O'Grady 2010). After an hour cells were washed and fixed with 3.7% paraformaldehyde for 10 minutes at room temperature then treated with 0.1% Triton X for 4 minutes. Cells were then blocked with 1% Bovine Serum Albumin for 20 minutes and stained with Texas-Red phalloidin stain (5 U/ml) for 20 minutes. Images were taken using fluorescent microscopy. Lamellipodia pixel area was measured using ImageJ software (National Institutes of Health, Bethesda, MD) as previously described (Schiller, Maniak, O'Grady

2010) and the average lamellipodia area from each image was statistically analyzed.

Statistical Analysis

Statistical significance was determined using an unpaired, two-tailed t-test (for single comparisons) or an ANOVA followed by Dunnett's test for single comparisons between treatment conditions and a common untreated control. A value of $p < 0.05$ was considered significant.

D. Results:

The effects of β-AR agonists on epithelial cell migration was studied using ECIS after wounding cells with a 6V pulse for 60 seconds applied at a frequency of 30 kHz. The stages of migration and impedance changes after wounding are shown in figure 1A-C. Note that complete wound closure occurred in 300 minutes after wounding (Fig 1C). The normalized impedance increase over time is shown as a continuous line tracing and was found to be nearly linear for NHBE cells (Fig 1D). The data points shown in figure 1D represent measurements of the fractional increase in area of the electrode that was covered by cells at specific time points during the 300 minute restitution period after wounding. This result indicated that continuous impedance measurements directly reflect the extent of cell confluence over the electrode surface over time.

Epithelial cells have been shown to express multiple β-ARs and to respond to selective β-AR agonists (Ghoghawala et al. 2008) Quantitative PCR

results shown in figure 2A and 2B demonstrates that both NHBE and hVE express β-AR with the highest levels of expression for β_1 and β_2 receptors. The C_t value for the β_3 receptor was >30 cycles in both NHBE and hVE cells, indicating relatively low levels of mRNA and protein expression relative to the β_1 and β_2 subtypes.

In migration experiments treatment of NHBE cells with the pan specific β-AR agonist epinephrine caused an increase in the time it takes for complete wound closure to occur, and when treated with a β_2 selective agonist (salbutamol) the time to complete wound closure was increased more than what was observed with epinephrine (Fig. 3A). In contrast, when cells were treated with a β_1 selective agonist (dobutamine), no significant change in migration was observed compared to untreated controls (Fig. 3B). Similar results were obtained for NE and dopamine when applied to hVE and hCE cells (Figs. 4A) To quantitatively analyze the effects of β_2-AR agonists on the migration behavior of the NHBE cells, measurements of the time required for the impedance to reach half maximal restitution ($Z_{0.5}$) was measured and the results reported in figure 3C. Values for $Z_{0.5}$ measurements were significantly increased in cells treated with epinephrine or salbutamol compared to untreated control conditions but this was not true for cells treated with dobutamine. Increases in time to wound closure for NE were also observed in hVE and hCE cells (Figs. 4C and 4D) and a comparable response to dopamine was recorded in hVE cells (Fig. 4C).

The fractional confluence per hour in NHBE cells that were stimulated with epinephrine or salbutamol were significantly different when compared to untreated control cells, whereas no difference was observed in dobutamine treated cells. (Fig 3D). Furthermore, there was no significant difference between the epinephrine and salbutamol treated conditions. This finding indicated that β_2-AR agonists increased the time to complete wound closure by slowing the migration rate over the entire duration of the restitution process. Similarly, the initial rate (between 0 and 4 hrs) of restitution in hVE cells was significantly reduced in response to NE and dopamine, this was also true for hCE cells treated with NE. Interestingly, the biphasic effects of NE and dopamine in hVE cells indicate that migration rate is initially suppressed by β-AR agonists, but then recovers to the control rate well before complete restitution is achieved (Figs. 4A). In contrast, the effect of NE in hCE cells appears to produce a delay in the onset of migration but shortly after migration begins, the rate is comparable to the untreated control condition (Fig. 4B). The addition of NE in both hVE and hCE cells caused a significant delay in wound recovery time (Fig. 4C and D) which is also represented in the measurements of confluence per hour (Fig 4 E and F).

To further establish the identity of the β-AR subtype responsible for the effects of epinephrine on cell migration, a series of experiments were performed using β-AR antagonists. Pretreatment of NHBE and hVE cells with 10μM propranolol completely rescued the effect of epinephrine on migration (Figs. 5 and 6) Analysis of the time required to reach half maximal restitution in NHBE

and hVE cells is presented in figures 5B and 6B. Additionally, we tested ICI-118551, a β_2 selective antagonist prior to epinephrine treatment. Figure 5A shows that ICI-118551 also rescues the effect of epinephrine on cell migration as well as the time to reach half maximal recovery (Figure 5B).

In Calu-3 cells the effect of Cl^- replacement with methane sulfonic acid on cell migration was studied using ECIS. Figure 7A shows that Cl^- replacement significantly decreases the rate of cell migration compared to control conditions. Also when cells are additionally treated with $CFTR_{inh}$-172 no further effect on cell migration was observed. This result indicated that the Cl^- replacement effect was dependent on the activity of CFTR. In order to further verify the pharmacological actions of $CFTR_{inh}$-172 on CFTR function a $CFTR_{inh}$-172 dose response relationship was determined and presented in 7B. The IC_{50} value was 3.91 μM, consistent with inhibition of CFTR channel activity. In addition, figure 7C compares the effects of CFTR silencing by RNAi (Palmer et al. 2006) and $CFTR_{inh}$-172 treated shALTR cells (cells expressing a non-CFTR targeting shRNA). Both conditions increased the time to reach complete restitution when compared to the shALTR cells alone. A summary of the $Z_{0.5}$ values for Cl^- replacement, $CFTR_{inh}$-172, and CFTR silenced cells showing significant increases in the time needed to achieve 50% restitution is presented in Figure 7D. Moreover, chloride replacement conditions with or without $CFTR_{inh}$-172 and CFTR silenced or $CFTR_{inh}$-172 treated shALTR cells were all shown to have significantly decreased fractional confluence per hour compared to untreated

controls indicating that the overall rates of migration were slower and that the increase in the time required for restitution was not the result of a delay in the initiation of migration.

The effects of pretreatment of NHBE cells with $CFTR_{inh}$-172 with and without 10μM epinephrine are presented in figure 8. The results show that cells treated with $CFTR_{inh}$-172 alone exhibited the same delay in restitution as cells treated with $CFTR_{inh}$-172 + epinephrine (Fig 8A). Similar results were obtained with CFTR silenced Calu-3 cells treated with 10μM salbutamol. A statistical analysis of the $Z_{0.5}$ values is reported in figure 8B. The observation that neither epinephrine nor salbutamol produced an additional reduction in cell migration following CFTR inhibition indicated that a reduction in CFTR activity over time was associated with chronic exposure to β-AR agonists.

The effect of the β-AR inverse agonist carvedilol on cell migration in NHBE and Calu-3 cells is presented in figure 9. In both cell systems carvedilol caused an increase in the required time for wound closure when compared to the untreated control condition and NHBE cells produced an effect similar to the β_2-AR agonist isotharine (Figure 9A and B). The time to half maximal restitution was significantly greater in cells treated with carvedilol and isotharine (Figure 9B). These findings indicate that G-protein activation in response to agonist binding is not required to reduce cell migration and that pathways associated with MAP kinase signaling or possibly β_2-AR down-regulation are involved. Figure 10A and B show the effects of CFTR inhibition with $CFTR_{inh}$-172 and 10μM epinephrine or

salbutamol on lamellipodia area in NHBE and Calu-3 cells. β-agonist induced inhibition of cell migration was associated with a decrease in the extent of lamellipodia protrusion, this effect was similar to what was observed in cells treated with $CFTR_{inh}$-172.

E. Discussion

Previous studies have shown that β_2-AR stimulation with isoproterenol leads to a decrease in migratory speed of both keratinocytes and corneal epithelial cells (Chen, Hoffman, Isseroff 2002; Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003) The authors reported that activation of the receptor resulted in dephosphorylation of extracellular signal-related kinase (ERK) and a decrease in the migration rate (Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003; Pullar et al. 2006). Stimulation of β_2-AR in both keratinocytes and corneal epithelial cells produced an increase in protein phosphatase 2A (PP2A) activity. The effect β-AR agonists have on migration and ERK phosphorylation was reversed when cells were pretreated with okadaic acid a PP2A inhibitor, suggesting that inhibition of migration was dependent on the PP2A pathway (Pullar, Chen, Isseroff 2003). It was later shown that treating cells with β-AR antagonists produced an opposite effect on both keratinocytes and corneal epithelial cells, increasing the rate of wound repair and promoting ERK phosphorylation (Pullar, Rizzo, Isseroff 2006; Pullar et al. 2007).

Although the effects of β-AR stimulation have been well studied in keratinocytes and corneal epithelial cells, the effects of β-AR stimulation on airway epithelial cell migration has not been extensively investigated. Results from the present study demonstrated that when NHBE and Calu-3 cells were stimulated with either epinephrine or salbutamol, a significant decrease in cell migration was observed. This effect however was not detected when cells were treated with a β_1-AR agonist dobutamine. These results are consistent with previous findings in keratinocytes and corneal epithelium where the actions of epinephrine were also mediated through β_2-ARs (Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003; Pullar et al. 2006). In studies performed by Pullar et al it was shown that β-AR antagonists accelerated wound repair by increasing the phosphorylation state of ERK (Pullar, Rizzo, Isseroff 2006; Pullar et al. 2007). Experiments with NHBE and Calu-3 cells pretreated with the panspecific β-AR antagonist propranolol or the β_2-AR antagonist ICI-118551 and then stimulated with epinephrine also showed no decrease in the cell migration rate. This phenomenon was also shown in hVE and hCE cells, indicating that inhibitory regulation of cell migration following β_2-AR stimulation is broadly observed in epithelial cells.

CFTR function was previously shown to be essential in the migration of airway epithelial cells (Schiller, Maniak, O'Grady 2010). Silencing the expression of CFTR or treating Calu-3 cells with $CFTR_{inh}$-172, a CFTR inhibitor, produced a significant decrease in the rate of cell migration. Similarly, when Calu-3 cells

expressing a non-CFTR targeting shRNA were treated with $CFTR_{inh}$-172 the effects on cell migration were comparable to CFTR silenced cells, causing delayed wound closure. To determine if the inhibitory effects of epinephrine on migration were linked to an effect on CFTR, experiments were conducted to establish whether inhibition of CFTR activity or protein expression would block any further decrease in migration evoked by epinephrine or salbutamol. The results indicated that the effects of epinephrine on migration were dependent on functional CFTR since no additive effects of β-AR agonists on migration were observed. There are several ways in which epinephrine could affect the availability or function of CFTR in airway epithelial cells. Previous studies by Naren et al showed that β_2-AR and CFTR are physically linked in the plasma membrane (Naren et al. 2003). It is known that epinephrine leads to G_s signaling causing an increase in cAMP production. (Benovic 2002; Han, Kommaddi, Shenoy 2013; Naren et al. 2003; Reiter and Lefkowitz 2006; Reiter et al. 2012; Shenoy et al. 2006; Shenoy and Lefkowitz 2011). This increase in cAMP production activates CFTR leading to Cl^- secretion. We showed that when there is no Cl^- transport, whether it is due to lack of Cl^- in the medium or an inability to transport Cl^-, for example due to limited expression or non-functional CFTR, a decrease in migration rate is observed. These results provide evidence that Cl^- transport may play an important role in wound repair.

It is known that epithelial cells respond to electric fields (EF) caused by disruption (wounding) of the epithelium. Cells respond to this field by moving

toward the cathode at the center of the wound, thus the electric field acts as a guidance cue to direct cell migration in a process that is called electrotaxis (Chiang, Robinson, Vanable 1992; Song et al. 2002; Sun et al. 2011; Zhao et al. 1996a; Zhao et al. 1996b)**.** CFTR appears to play an essential role in producing this wound current and guidance cue, which affects lamellipodia protrusion (Schiller, Maniak, O'Grady 2010; Sun et al. 2011). Interestingly, measurements of lamellipodia area after treatment with salbutamol or epinephrine showed a significant decrease compared to the untreated control condition, which was nearly identical to the effect of $CFTR_{inh}$-172 on lamellipodia area. These results support the hypothesis that chronic exposure to β_2-AR agonists produce a decrease in CFTR function over the time course of the wound healing experiment and that this results in a reduced wound current and loss of directional control over the migration process. Previous studies reported in Chapter 2 of this thesis indicate that chronic exposure to carvedilol induces down-regulation of the β_2-AR receptor and a decrease in the level of CFTR expression in the apical membrane of Calu-3 cells, which is also consistent with this hypothesis.

The effects of long acting β_2-AR agonists have been shown to have increased negative effects with chronic use. These drugs are usually prescribed over the lifetime of individuals that suffer from obstructive airway disease (Kew, Dias, Cates 2014; Saguil and Garcia 2014) This pattern of drug use can lead to decreased effectiveness of β-agonists on the airways which can increase the number of exacerbations as well as the severity of such symptoms (Page and

Spina 2006; Walker et al. 2011; Wasilewski, Lougheed, Fisher 2014). This evidence suggests a potential need for change in the use of β_2-AR agonists in treating obstructive airway diseases and investigate other possibilities. Bias ligands are one potential solution for new drug therapies although their effectiveness for symptom relief in individuals suffering from an obstructive airway disease has not been greatly studied. Currently carvedilol, a β_2-AR inverse bias ligand is used in patients with heart disease to promote vasodilation and decrease heart rate (Bristow et al. 1996; Kveiborg et al. 2007; Vanderhoff, Ruppel, Amsterdam 1998). Although inverse ligands have shown to be beneficial for cardiac patients, the use of them in the airways is limited. Treatment of NHBE or Calu-3 cells with carvedilol produces a significant decrease in rate of wound closure which is very similar to the effect of epinephrine or salbutamol. This effect on migration is likely due to a conformational change in the receptor preventing activation of G_s signaling and activating the β-arrestin signaling pathway (Kim et al. 2008; Shenoy and Lefkowitz 2011; Wisler et al. 2007). This pathway, which has been discussed in Chapter 2, suggests that MARCH2, an ubiquitin ligase, is responsible for promoting internalization of the β_2-AR-CFTR signaling complex (Han et al. 2012; Wisler et al. 2007). This would lead to a decrease in apical membrane CFTR expression and reduce the rate of cell migration during wound repair.

In summary, these experiments have shown that β_2-AR stimulation leads to a significant decrease in the rate of cell migration of airway epithelial cells and

this effect is rescued when cells are pretreated with β-AR antagonists. We hypothesize that epinephrine caused down-regulation of the β_2-AR leading to a decrease in G_s signaling which slows migration speed and increases the time required for wound closure. We have also shown that treating cells with the inverse ligand carvedilol also reduces the rate of wound closure, consistent with down-regulation of the β_2-AR signaling complex. The need for new drug development to treat the symptoms of individuals with obstructive airway disease with no negative impact on airway restitution is essential. Bias ligands may be the answer. A bias ligand that activates only the G_s signaling arm of GPCR would promote smooth muscle relaxation and CFTR activation. These drugs would avoid β-arrestin dependent signaling, preventing the negative downstream effects on migration.

Table 1: Human primer sets used for qRT-PCR in NHBE cells.

Primer	Sequence (5'-3')
GAPDH F	TGGAAATCCCATCACCATCT
GAPDH R	TTCACACCCATGACGAACAT
β-Actin F	TCCCTGGAGAAGAGCTACGA
β-Actin R	AGCACTGTGTTGGCGTACAG
β_1-AR F	CAATGACACACAGGGTCTCG
β_1-AR R	CAGACGCTCACCAACCTCTT
β_2-AR F	TGCTATGCCAATGAGACCTG
β_2-AR R	TCCACCTGGCTAAGGTTCTG
β_3-AR F	TGACCAACGTGTTCGTGACT
β_3-AR R	GTTGGTCACAGCCAGGTAGC

Figure 1

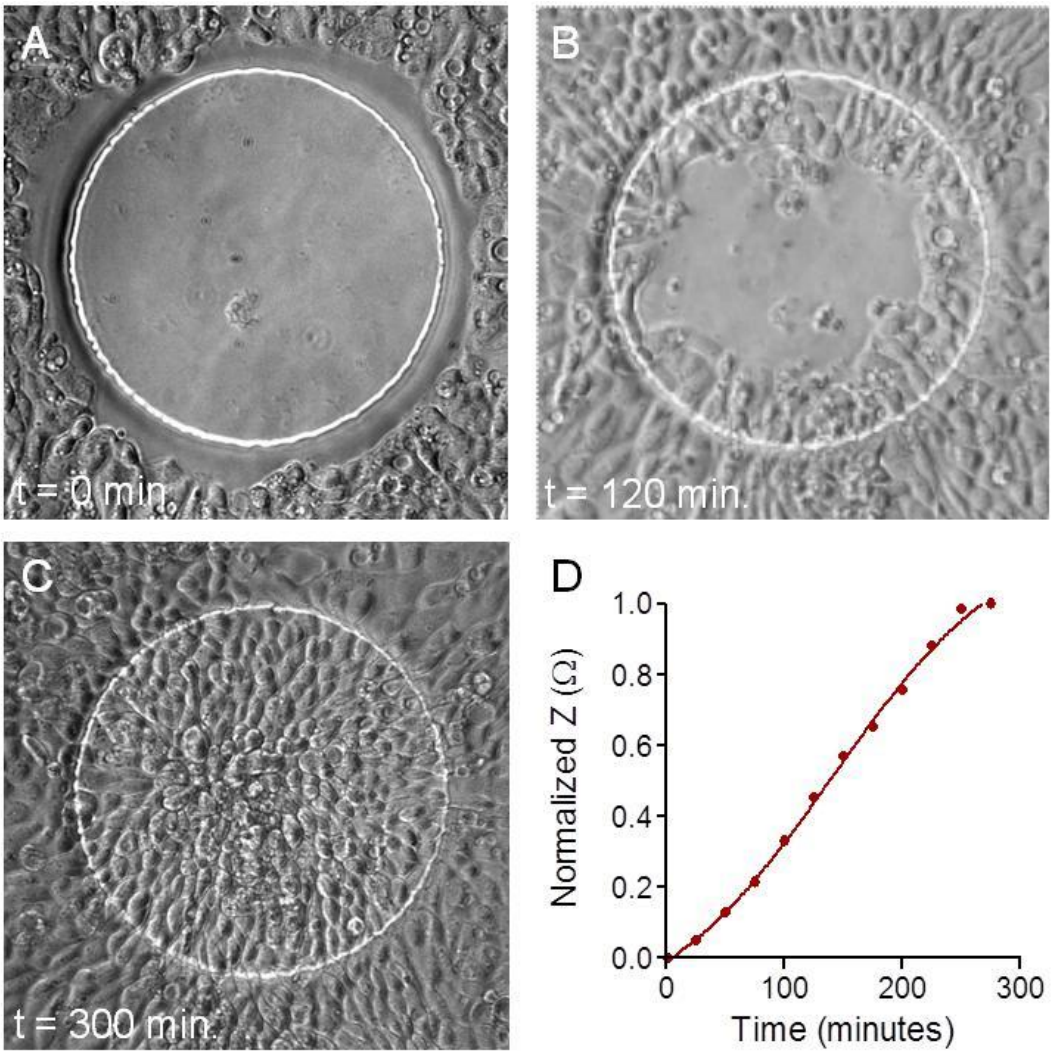

Figure 1: Impedance is a measure of cell coverage on electrodes during migration. Images of NHBE cells on electrodes at the different migration stages: **A**: Immediately after wounding; **B**: 200 minutes and **C**: 300 minutes after wounding. **D:** Solid line: The averaged normalized impedance over time during wound closure from NHBE cells. Data points: Normalized fractional coverage of the electrode by the NHBE cells.

Figure 2

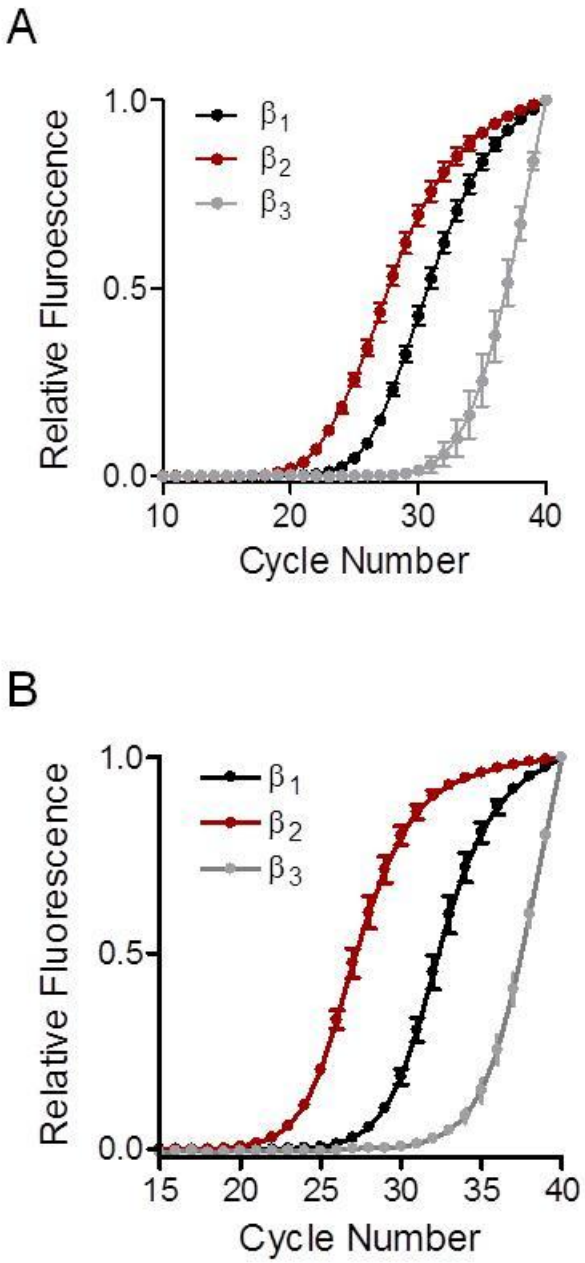

Figure 2: β_2-AR is the most prevalent β-AR in NHBE and hVE cells **A**: QRT-PCR amplification plot showing relative fluorescence vs. Cycle number for hVE cells (n=6). **B**: QRT-PCR amplification plot showing relative fluorescence vs. Cycle Number for NHBE cells (n=6).

Figure 3

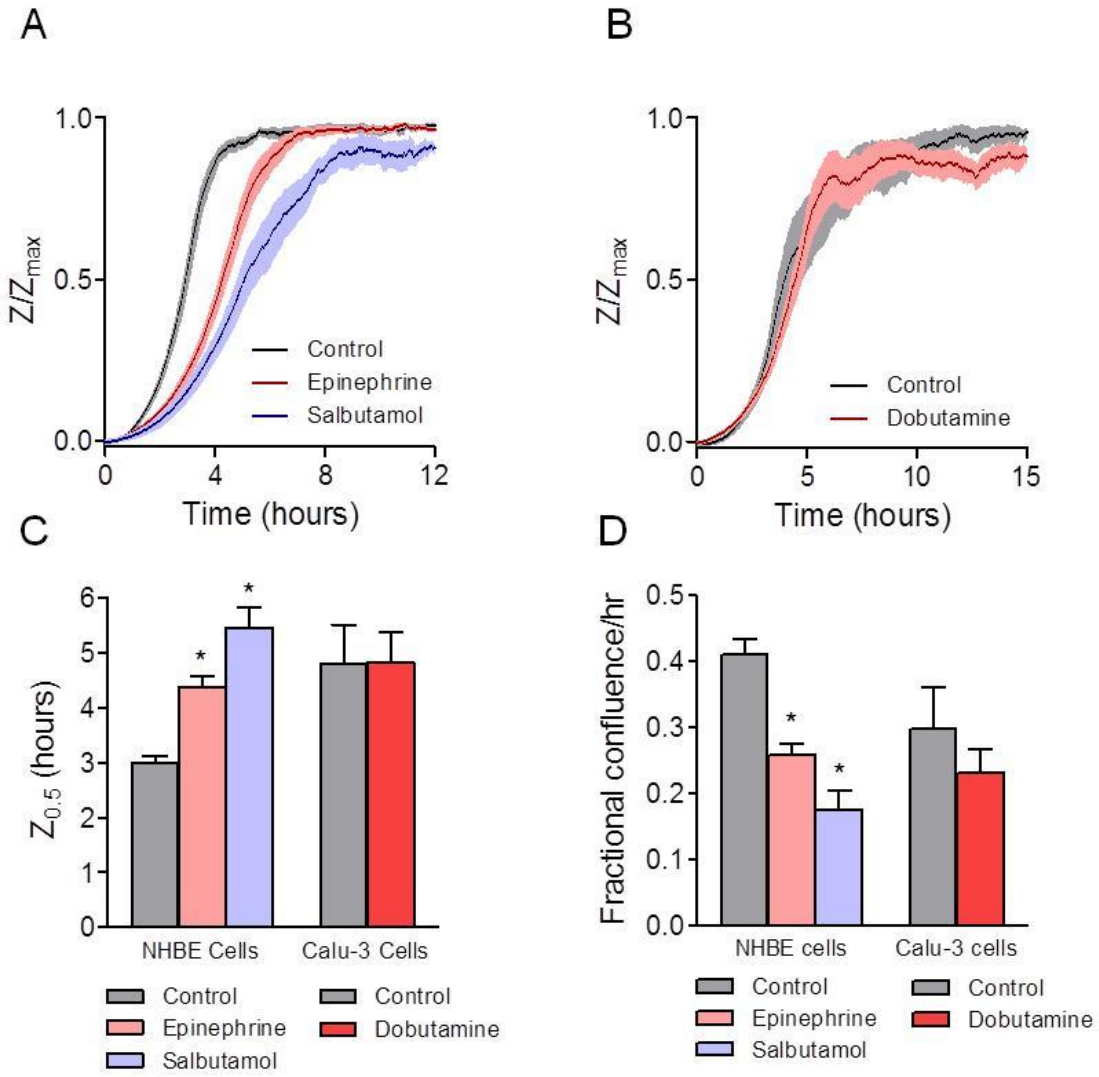

Figure 3: Effects of β-AR agonists on migration rate of NHBE cells. **A:** The averaged change in impedance relative to the maximum impedance (normalized impedance change) during wound closure of NHBE cells treated with vehicle (DMSO; black line, n=8), epinephrine (10μM epinephrine + DMSO; red line, n=8) or salbutamol (10μM salbutamol; blue line, n=8) **B:** The normalized impedance change during wound closure of NHBE cells treated with vehicle (DMSO; grey bar, n=8) or dobutamine (red line, n=8). **C:** The averaged time to reach half maximal restitution of NHBE cells treated with vehicle (DMSO; grey bar, n=8), epinephrine (10μM epinephrine + DMSO; pink bar, n=8), salbutamol (10μM salbutamol; blue bar, n=8), control (grey bar, n=8) or dobutamine (10μM dobutamine; red bar, n=8). **D:** The fractional confluence per hour of NHBE and Calu-3 cells treated with vehicle (DMSO; grey bar, n=8), epinephrine (10μM epinephrine + DMSO; pink bar, n=8), salbutamol (10μM salbutamol; blue bar, n=8), control (grey bar, n=8) or dobutamine (10μM dobutamine; red bar, n=8).

Figure 4

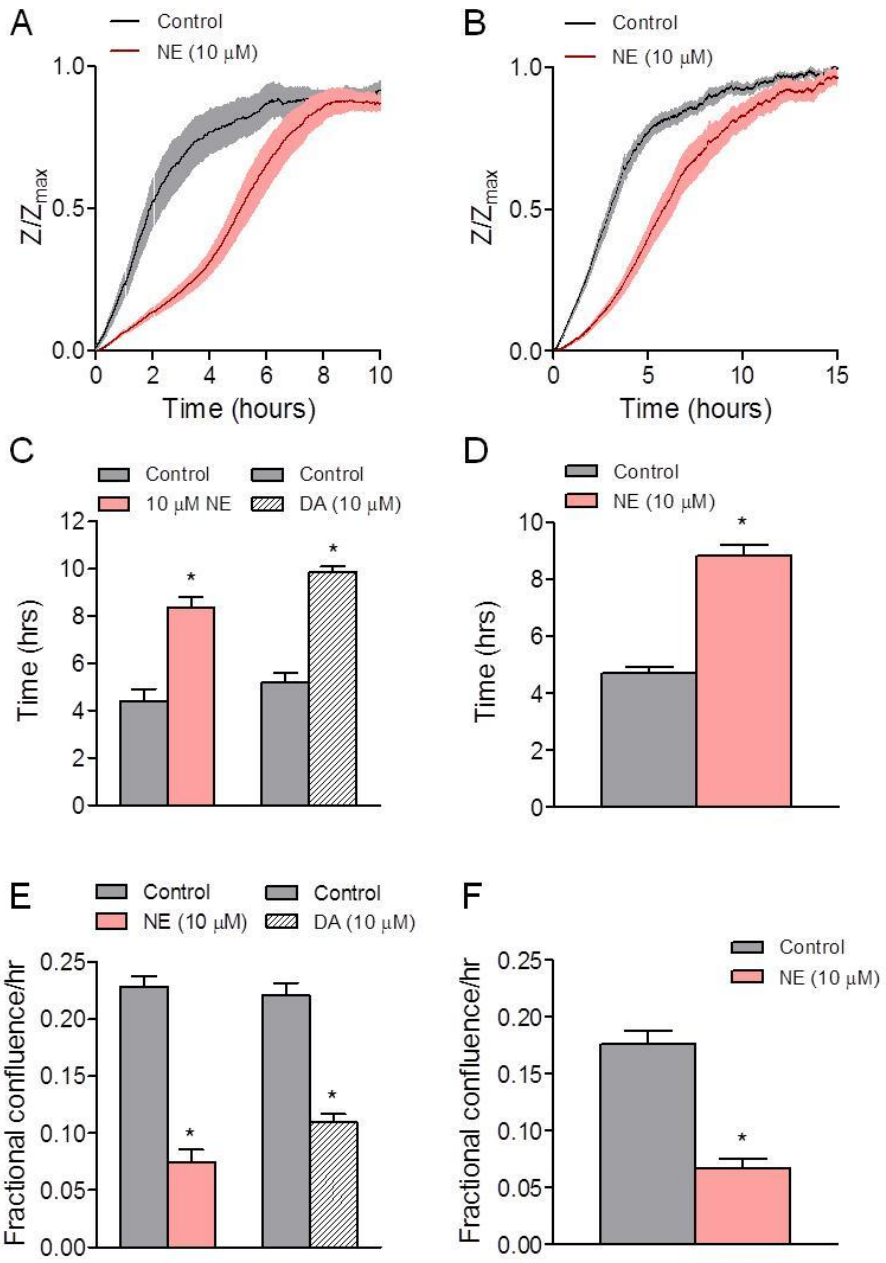

Figure 4: Effects of norepinephrine on migration rate of hVE and hCE cells. **A:** The averaged normalized impedance change during wound closure of hVE cells treated with control (black line, n=8), or norepinephrine (red line, n=8) **B:** The averaged normalized impedance change during wound closure of hCE cells treated with control (black line, n=8), or norepinephrine (red line, n=8) **C:** The averaged time to reach half maximal restitution of hVE cells treated with control (grey bar, n=8), norepinephrine (red bar, n=8) or dopamine (striped bar, n=8). **D:** The averaged time to reach half maximal restitution of hCE cells treated with control (grey bar, n=8), or norepinephrine (red bar, n=8) **E:** The fractional confluence per hour of hVE cells treated with control (grey bar, n=8) , norepinephrine (red bar, n=8) or dopamine (striped bar, n=8). **F:** The fractional confluence per hour of hCE cells treated with control (grey bar, n=8), or norepinephrine (red bar, n=8).

Figure 5

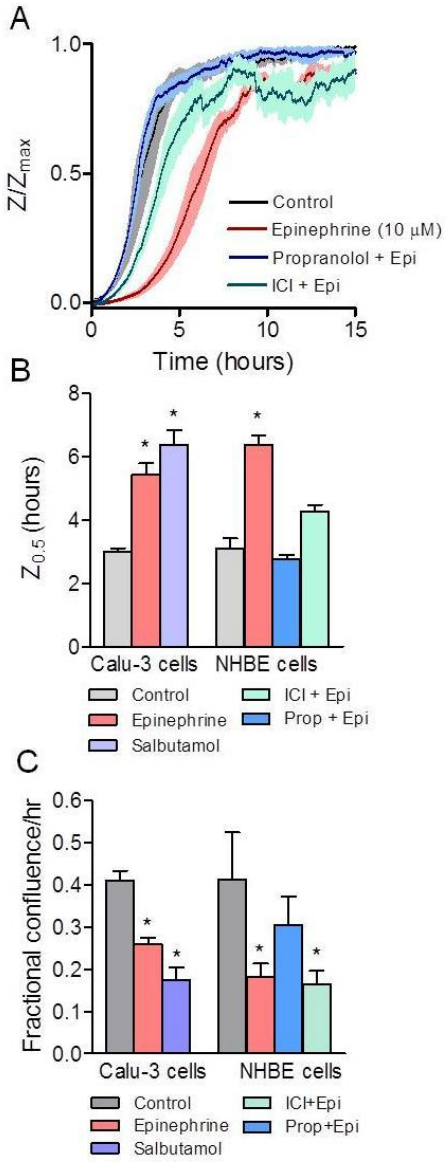

Figure 5: Effects of β-AR agonists and antagonists on migration rate of NHBE cells over time. **A:** The averaged normalized impedance change during wound closure of NHBE cells treated with vehicle (DMSO; black line, n=4), epinephrine (10μM epinephrine + DMSO; red line, n=5) propranolol + epinephrine (10μM propranolol + 10μM epinephrine + DMSO; blue line, n=6) or ICI-118551 + epinephrine (1μM ICI + 10μM epinephrine; green line, n=4). **B:** The averaged time to reach half maximal restitution of NHBE cells treated with vehicle (DMSO; grey bar, n=4) epinephrine (10μM epinephrine + DMSO; red bar, n=5), salbutamol (salbutamol; purple bar, n=8) propranolol + epinephrine (10μM propranolol + 10μM epinephrine + DMSO; blue bar, n=6) or ICI-118551 + epinephrine (1μM ICI + 10μM epinephrine; green bar, n=4). **C:** The fractional confluence per hour of NHBE cells treated with vehicle (DMSO; grey bar, n=4) epinephrine (10μM epinephrine + DMSO; red bar, n=5), salbutamol (salbutamol; purple bar, n=8) propranolol + epinephrine (10μM propranolol + 10μM epinephrine + DMSO; blue bar, n=6) or ICI-118551 + epinephrine (1μM ICI + 10μM epinephrine; green bar, n=4).

Figure 6

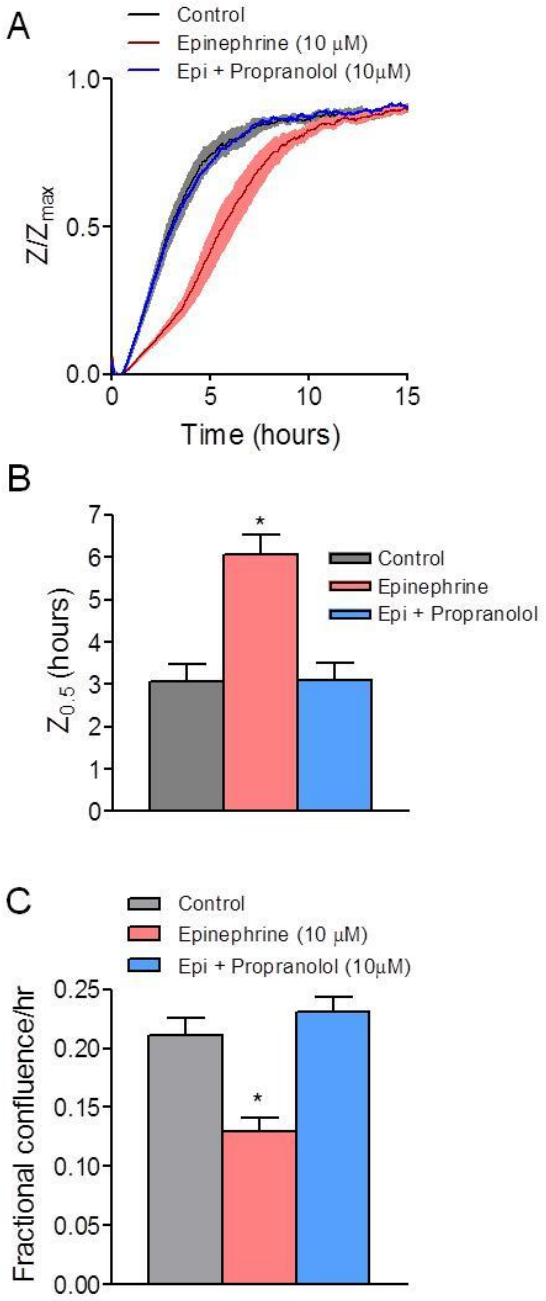

Figure 6: Effects of β-AR agonists and antagonists on migration rate of hVE cells over time. **A:** The averaged normalized impedance change during wound closure of hVE cells treated with control (black line, n=10), epinephrine (10μM epinephrine; red line, n=11) or propranolol + epinephrine (10μM propranolol + 10μM epinephrine; blue line, n=10). **B:** The averaged time to reach half maximal restitution hVE cells treated with control (grey bar, n=10), epinephrine (10μM epinephrine; red bar, n=11) or propranolol + epinephrine (10μM propranolol + 10μM epinephrine; blue bar, n=10). **C:** The fractional confluence per hour of hVE cells treated with control (grey bar, n=10), epinephrine (10μM epinephrine; red bar, n=11) or propranolol + epinephrine (10μM propranolol + 10μM epinephrine; blue bar, n=10).

Figure 7

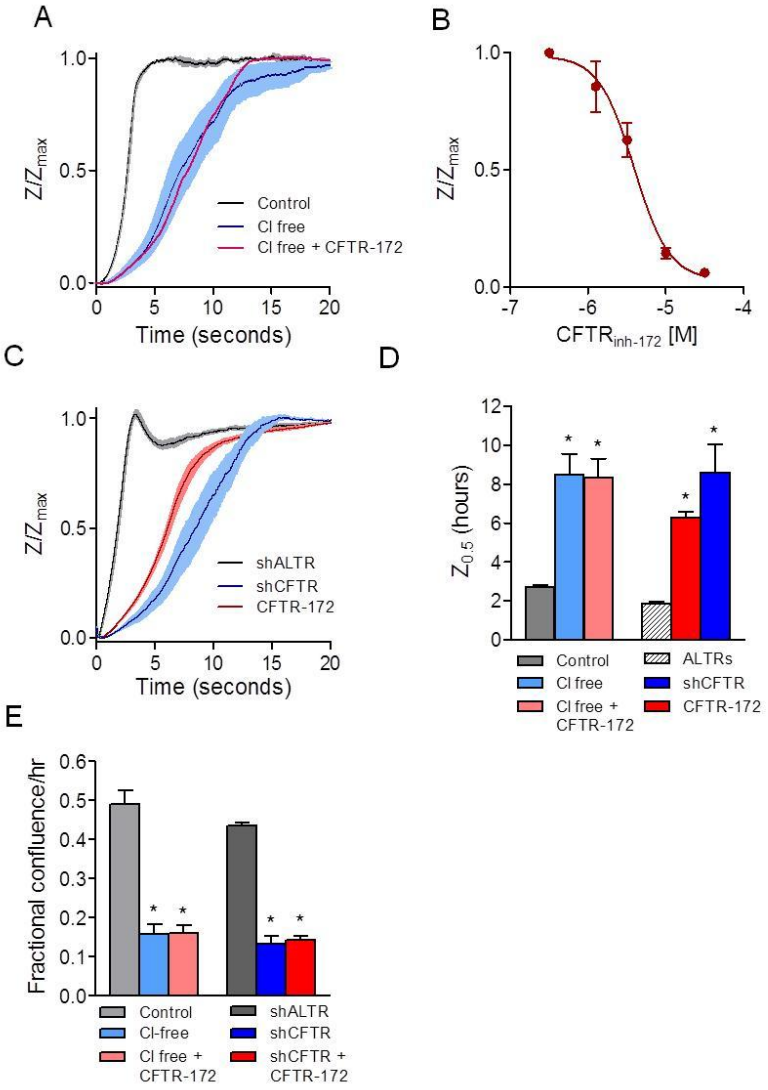

Figure 7: The importance of CFTR on the migration rate of Calu-3 cells. **A:** The averaged normalized impedance change during wound closure of Calu-3 cells treated with vehicle (DMSO; black line, n=4), chloride free media (DMSO; blue line, n=10), or chloride free with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; red line, n=5). **B:** The averaged normalized impedance change over time of a dose response to $CFTR_{inh}$-172 in Calu-3 cells. **C:** The averaged normalized impedance over time during wound closure of Calu-3 ALTR cells (DMSO; black line, n=7), shCFTR Calu-3 cells (DMSO; blue line, n=4) or Calu-3 ALTRs with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; red line, n=8). **D:** The averaged time to reach half maximal restitution of Calu-3 treated with vehicle (DMSO; light grey bar, n=4), chloride free media (DMSO; light blue bar, n=10), chloride free with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; pink bar, n=5), Calu-3 ALTR cells (DMSO; striped bar, n=7), shCFTR Calu-3 cells (DMSO; blue bar, n=4) or Calu-3 ALTRs with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; red bar, n=8).**E:** The fractional confluence per hour of Calu-3 treated with vehicle (DMSO; light grey bar, n=4), chloride free media (DMSO; light blue bar, n=10), chloride free with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; pink bar, n=5), Calu-3 ALTR cells (DMSO; dark grey bar, n=7), shCFTR Calu-3 cells (DMSO; blue bar, n=4) or Calu-3 ALTRs with $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; red bar, n=8).

Figure 8

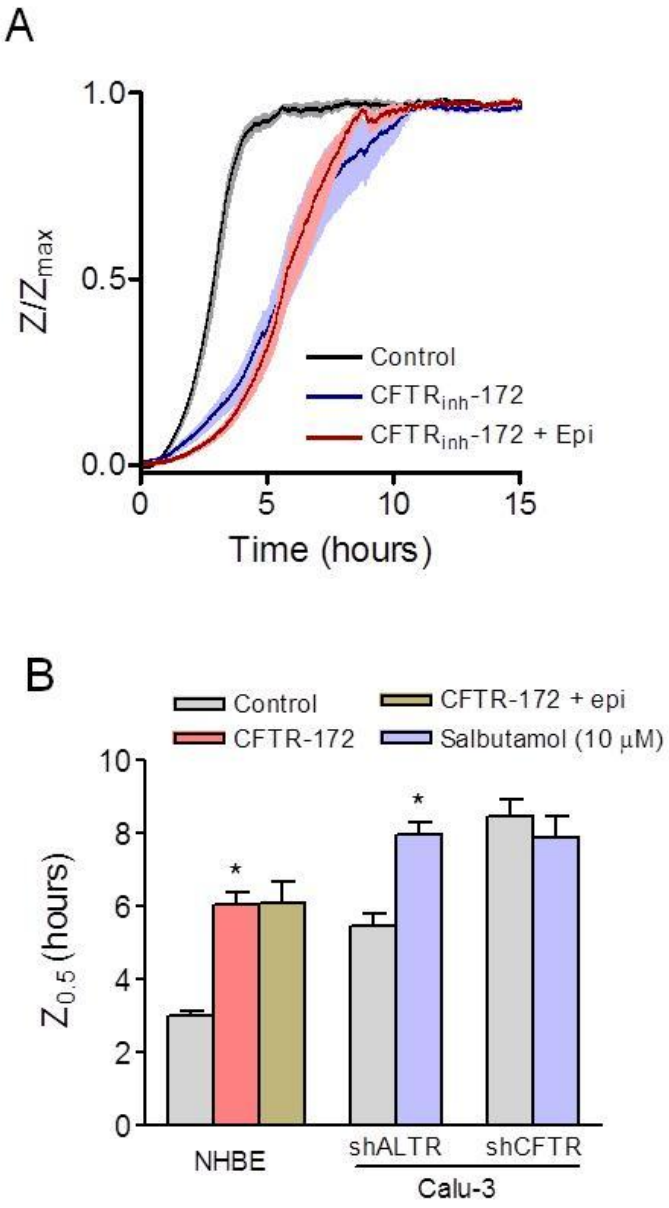

Figure 8: Effects of epinephrine or salbutamol on Calu-3 that were pretreated with $CFTR_{inh}$-172 or CFTR silenced. **A:** The averaged normalized impedance change during wound closure of Calu-3 cells treated with vehicle (DMSO; black line, n=8), $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; blue line, n=8) or $CFTR_{inh}$-172 + epinephrine (20μM $CFTR_{inh}$-172 + 10μM epinephrine; red line, n=8). **B:** The averaged time to reach half maximal restitution of NHBE treated with vehicle (DMSO; grey bar, n=8), $CFTR_{inh}$-172 (20μM $CFTR_{inh}$-172; red bar, n=8), $CFTR_{inh}$-172 + epinephrine (20μM $CFTR_{inh}$-172 + 10μM epinephrine; green bar, n=8) shALTR cells treated with vehicle (DMSO; grey bar, n=6), and shCFTR cells treated with vehicle (DMSO; grey bar, n=7), shALTR cells treated with salbutamol (10μM salbutamol; blue bar, n=6), shCFTR cells treated with salbutamol (10μM salbutamol; blue bar, n=6).

Figure 9

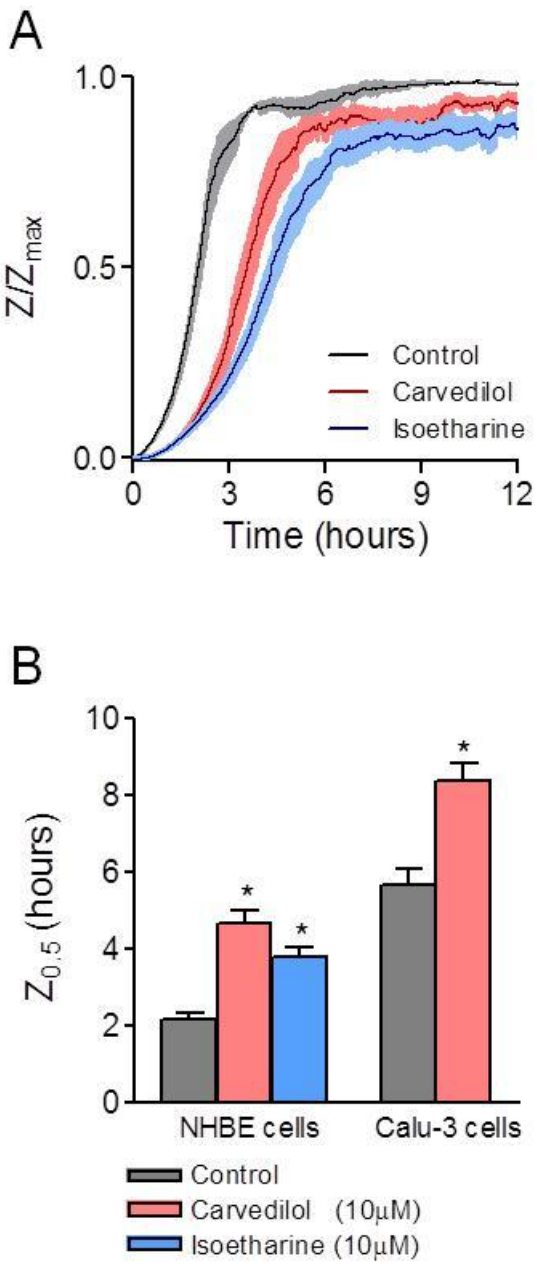

Figure 9: Effects of β-AR inverse ligands on migration rate of Calu-3 and NHBE cells over time. **A:** The averaged normalized impedance change during wound closure of NHBE cells treated with vehicle (DMSO; black line, n=6), carvedilol (10μM carvedilol; red line, n=8) or isoetharine (10μM isoetharine; blue line, n=7). **B:** The averaged time to reach half maximal restitution of Calu-3 and NHBE cells treated with vehicle (DMSO; grey bar, NHBE n=6 and Calu-3 n=11), carvedilol (10μM carvedilol; pink bar, NHBE n=8 and Calu-3 n=9) or isoetharine (10μM isoetharine; blue bar, n=7).

Figure 10

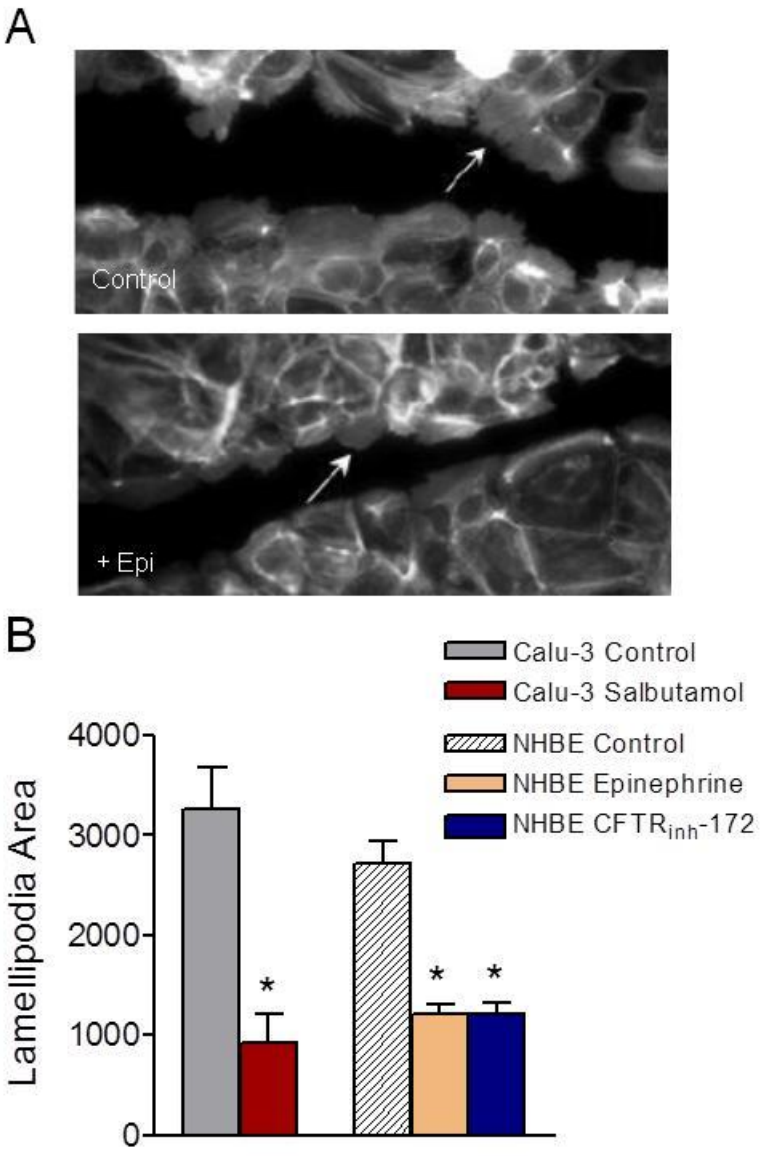

Figure 10: Effects of epinephrine and salbutamol on lamellipodia protrusion in NHBE and Calu-3 cells. **A:** An image of lamellipodia protrusion in control (top) and epinephrine treated (bottom) cells. **B:** Average pixel area of lamellipodia per NHBE cell treated with vehicle (DMSO; grey bar, n=6 fields), epinephrine (10μM epinephrine + DMSO; pink bar, n=5 fields) or $CFTR_{inh}$-172 (blue bar, n=5 fields) and Calu-3 cells treated with vehicle (DMSO; grey bar, n=5 fields) and 10μM salbutamol (red bar, n=4 fields).

Chapter 4

Conclusions

A. Conclusion of Thesis:

Previous studies have shown the importance of CFTR and β_2-AR agonists on the migration of airway epithelial cells (Chen, Hoffman, Isseroff 2002; Pullar et al. 2006; Pullar et al. 2007; Schiller, Maniak, O'Grady 2010; Sun et al. 2011). Although β_2-AR agonists are effective in providing symptom relief in individuals with obstructive airway disease these drugs have been shown to decrease the ability of the airway epithelium to effectively repair itself following injury (Cockcroft and Sears 2013; Page and Spina 2006; Spina 2014). These studies showed that since the introduction of inhaled β-AR agonists there have been problems in determining the most effective dose with least amount of risk (Cockcroft and Sears 2013; Page and Spina 2006). In the 1960s some countries such as the United Kingdom and Australia were using an increased dose of isoproteranol compared to the United States and it was shown that the use of this inhaled non-specific β-AR agonist increased the number of asthma related deaths (Cockcroft and Sears 2013; Page and Spina 2006). This was seen again in New Zealand with fenoterol a short acting β agonist and in 2000 the Salmeterol Multicenter Asthma Research Trial (SMART) showed the use of salmeterol lead to an increase in asthma mortality (Cockcroft and Sears 2013; Nelson et al. 2006; Page and Spina 2006). It is suggested that the chronic use of β-AR agonists causes an increase in medication tolerance, responsiveness of the airways to allergins, and inflammation. It is also thought that when these medications are used, they alleiviate the initial asthmaic episode leading to

inflammation masking of the later stages of the asthmatic response which potentially leads to increased inflammation and epithelial damage over time (Cockcroft and Sears 2013; Page and Spina 2006). The continual damage to the airway over time is thought to be the culprit of β-AR agonist related deaths.

1. Adrenergic receptors and mucociliary clearance

Earlier studies by Naren et al. (2003) showed that apical β_2 ARs are coupled to CFTR by adapter proteins that form a scaffold for PKA, ensuring that the cAMP-dependent kinase is positioned in close proximity to phosphorylation sites within the regulatory domain of CFTR (Figure 1).

Figure 1

β_2-AR-CFTR signaling complex

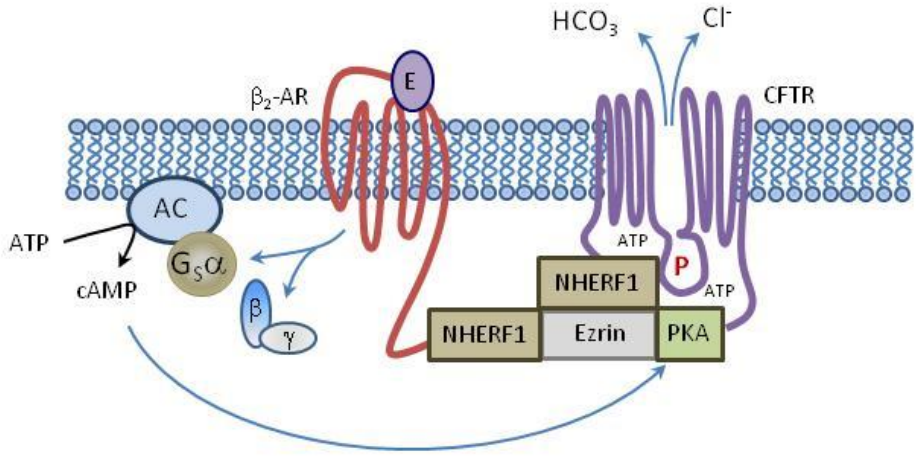

Figure 1: A model of the CFTR-β2-AR signaling complex connected by adapter proteins NHERF1, EZRIN and PKA in the apical membrane.

Data presented in Chapter 3 showed that stimulating β_2-ARs on the apical membrane with epinephrine produces an initial increase in anion secretion that is then significantly diminished after continuous agonist exposure. Stimulation of the receptor with epinephrine leads to the activation of G-protein signaling, generation of cAMP and subsequent activation of PKA. This kinase then phosphorylates CFTR causing the channel to open and allowing Cl^- to exit the cell across the apical membrane. With continuous agonist exposure however, phosphorylation of the receptor by GRK occurs, which facilitates binding of β-arrestin and down-regulation of the receptor. Biotynylation experiments in chapter 2 revealed that β_2-AR disappearance from the apical membrane is nearly complete within 30 minutes of continuous treatment with epinephrine or carvedilol. β_2-AR desensitization is thought to be responsible for the decrease in anion secretion that occurs with increasing time of agonist exposure. Previous studies have shown that activation of β-arrestin signaling after agonist binding leads to downstream signaling through MAPK activation as well as recruitment of Nedd4 an E3 ubiquitin ligase responsible for ubiquitination of proteins to promote protein trafficking to lysosomes (Han, Kommaddi, Shenoy 2013; Shenoy et al. 2008; Shenoy and Lefkowitz 2011). However once the receptor is internalized, deubiquitinases (USP20 and USP33) can associate with the β_2-AR and prevent degradation by deubiquinating the receptor, promoting receptor recycling (Berthouze et al. 2009; Han, Kommaddi, Shenoy 2013). Furthermore studies have shown that cAMP production after a β_2-agonist binds to the β_2-AR leads to

CFTR activation and phosphorylation of CFTR which is thought to cause the β_2-AR-CFTR signaling complex to dissociate (Naren et al. 2003). Thus the β_2-AR and CFTR are internalized and recycled or degraded separately.

When the β_2-AR was stimulated with the inverse ligand carvedilol, the immediate response was different from the effect observed with epinephrine. Carvedilol stimulation caused a decrease in basal CFTR-dependent anion secretion, consistent with an inverse ligand (Parra and Bond 2007), initiated conformational change of the receptor that promotes β-arrestin signaling while preventing G_s mediated signaling (Andresen 2011; Han et al. 2012; Kim et al. 2008; Kim et al. 2005; Shenoy and Lefkowitz 2011; Wisler et al. 2007). This leads to desensitization of the receptor (Tilley 2011) which in turn would decrease the ability of CFTR to be activated through β_2-AR activation. Figure 2 shows pathways that are activated by either epinephrine or carvedilol binding to the β_2-AR.

Note that epinephrine activates both G-protein and β-arrestin signaling pathways. In contrast, carvedilol does not activate G-protein signaling, but does activate β-arrestin mediated receptor desensitization and activation of MAPK dependent signaling. By blocking G_s signaling carvedilol prevents USP20 and USP33 from being activated therefore preventing the ability for the receptor to be deubiquinated and targeted for recycling (Berthouze et al. 2009; Han et al. 2012). Furthermore it has been shown that carvedilol blocks β_2-AR association with Nedd4 (Han et al. 2012; Wisler et al. 2007). Instead the receptors associate with

MARCH2 another E3 ubiquitin ligase that is recruited and activated upon carvedilol binding (Han et al. 2012) (figure 3). This E3 ligase also promotes receptor targeting to lysosomes resulting in degradation (Han et al. 2012; Wisler et al. 2007).

Figure 2

G protein and β-arrestin signaling

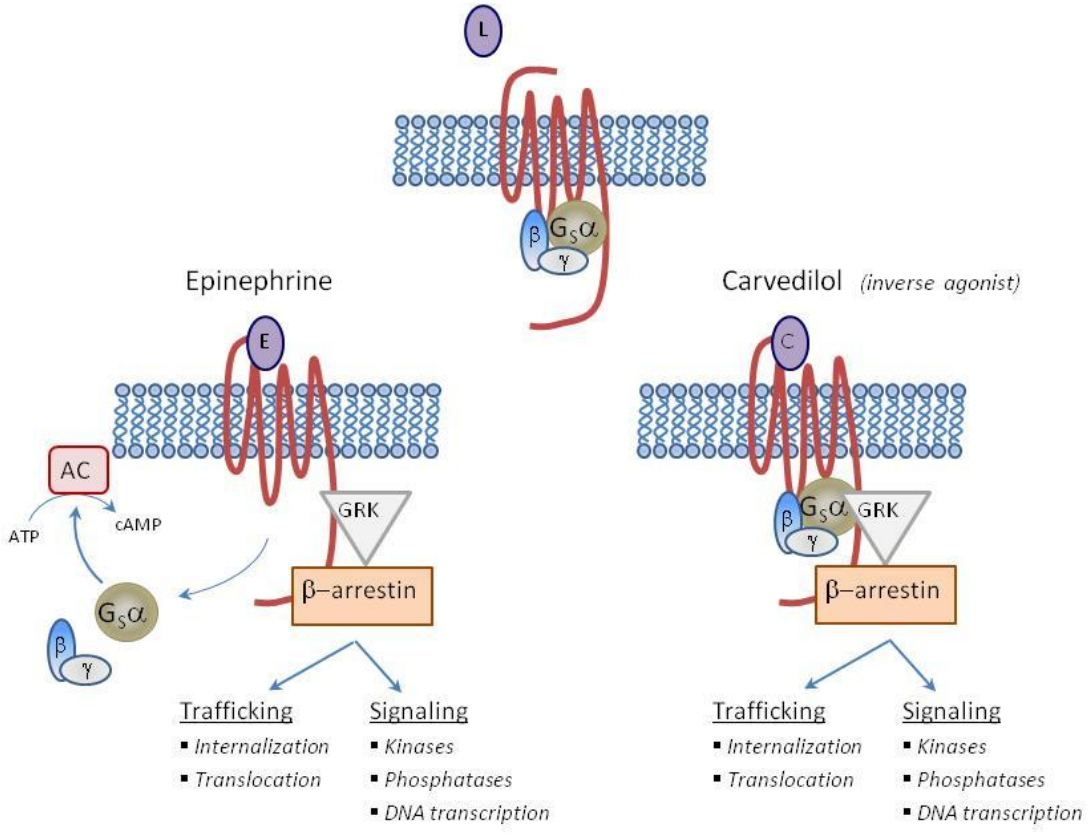

Figure 2: Effects of β_2-AR stimulation with epinephrine or carvedilol on signaling pathways. β2-AR causes G-Protein disassociation, activation of adenylyl cyclase leading to the production of cAMP. G-Protein Receptor Kinase (GRK) phosphorylates the C-termial tail of the receptor recruiting β-arrestin which promotes internalization and initiation of additional signaling molecules. Carvedilol blocks G-Protein dissociation and promotes β-arrestin signaling.

Figure 3

Carvedilol effects on β_2-AR signaling

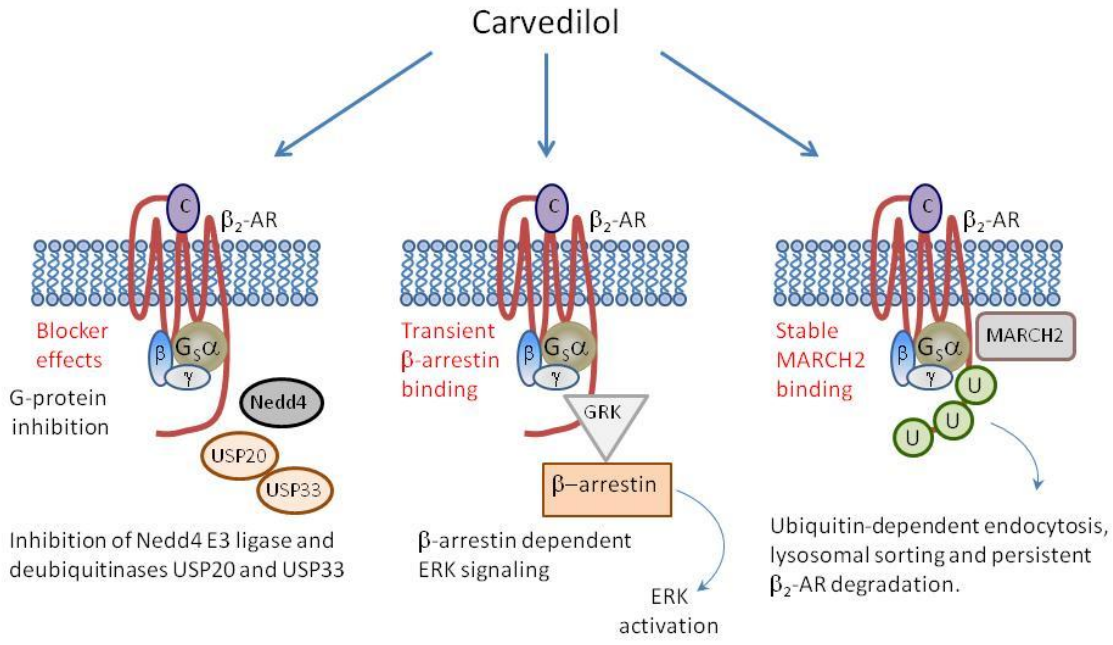

Figure 3: The effects of carvedilol after binding to the β_2-AR involves, blocking G_s signaling, inhibitng Nedd4 and USP20 and USP30 activation, initiating β-arrestin signaling, and triggering endocytosis via MARCH2 ligase.

An important result from chapter 2 was the observation that within minutes following treatment with carvedilol, basal anion secretion was inhibited and over a time period of 30 minutes, CFTR expression in the plasma membrane decreased by nearly 60% whereas epinephrine did not significantly alter the level of channel expression in the apical membrane over the same time period. The short-term response to carvedilol was likely due to inhibition of G_s-dependent signaling that results from the conformation change that occurs in the β_2-AR induced by carvedilol. In addition this change in receptor conformation may also

prevent uncoupling of the β_2-AR-CFTR signaling complex, suggesting the possibility that desensitization involves retreval of both β_2-AR and CFTR together from the apical membrane (Figure 4). As previously mentioned, uncoupling of CFTR from the β_2-AR appears to be dependent upon CFTR phosphorylation by PKA, therefore inhibition of G_s-signaling and dephosphorylation of the channel by PP2A could preseve NHERF-mediated scaffolding interactions between the two proteins as shown in Figure 1.

Figure 4

β_2AR-CFTR signaling complex in airway epithelial cells

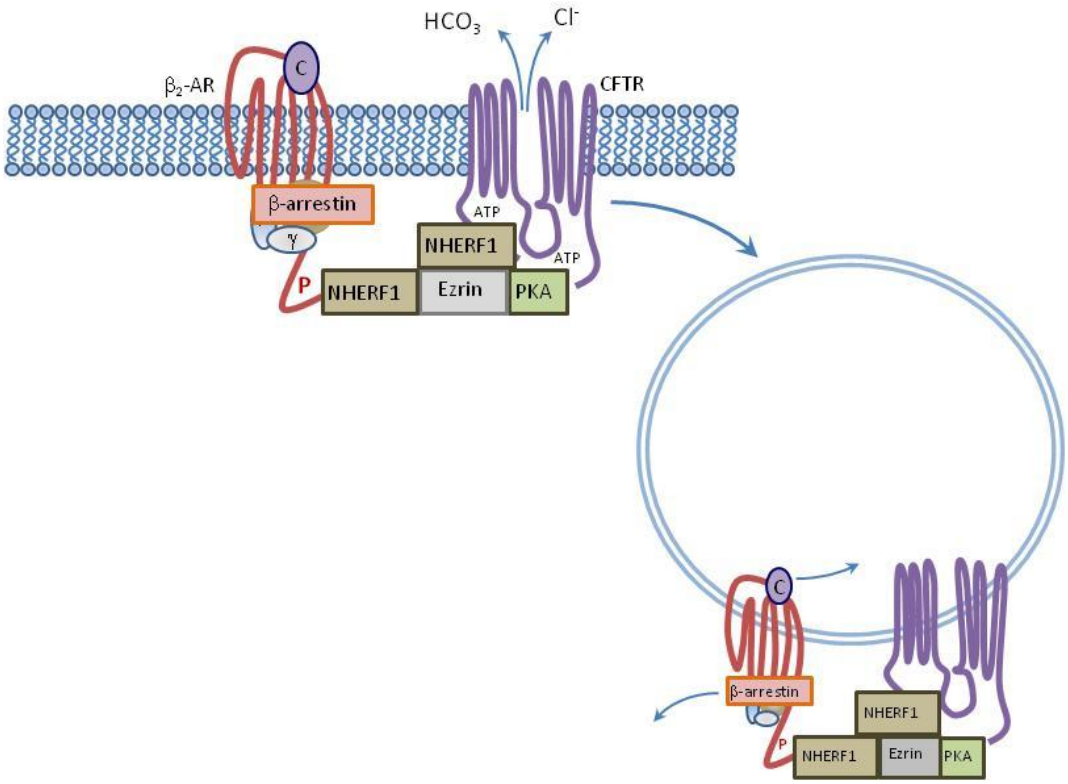

Figure 4: β_2-AR-CFTR signaling complex located in the apical membrane of airway epithelial cells is internalized after β_2-AR stimulation with carvedilol.

2. Adrenergic regulation of airway epithelial cell migration and wound repair

It has been previously suggested that β-agonists activate ERK-dependent signaling in HEK293 (human embryonic kidney cells), COS-7 cells (monkey fibroblast like cells) and cardiac myocytes studying cardiac hypertrophy (Schmitt and Stork 2000; Zou et al. 2001). These studies showed that treating cells with isoproterenol leads to β_2-AR activation resulting in ERK phosphorylation which was blocked with alpreolol a β-AR antagonist (Schmitt and Stork 2000). It is also known that ERK activation can promote cell migration which has been shown in osteoblasts and fibrosarcoma cells (Lai et al. 2001; Webb, Nguyen, Gonias 2000). Studies used dominate negative mutants of ERK as well as ERK inhibitors to show that ERK is important in migration, both the dominant negative mutant and cells treated with ERK inhibitors blocked cell migration (Lai et al. 2001).

As previously stated ERK plays an important role in cell migration, and does so by interacting with several other proteins involved in the protrusion, adhesion and retraction processes of migration. Myosin light chain kinase (MLCK) is one of these proteins. It has been shown that ERK is important in the activation of MLCK and MLC which play a role in the turnover of focal adhesions and protrusions at the leading edge of migrating cell (Huang, Jacobson, Schaller 2004). Calpins, Ca^{2+} activated enzymes, are also activated by ERK in low calcium conditions, and are important in focal adhesion disassembly and cytoskeletal changes needed for cell migration such as trailing edge detachment from ECM and leading edge protrusions (Campbell and Davies 2012; Huang,

Jacobson, Schaller 2004). Focal adhesion kinases (FAKs) also are important in cell migration. Studies have shown that FAKs are important in the phosphorylation of paxillin-kinase linker proteins which are known to interact with paxillin, promoting focal adhesion stimulation and cell polarity both of which are important in the process of migration (Yu, Deakin, Turner 2010) and ERK is responsible for their activation as well as the activation of FAK-Paxillin association (Huang, Jacobson, Schaller 2004). It has recently been shown that ERK is also responsible for phosphorylating histone deacetylase 6, a migration promoter that associates with microtubules and causes deacetylation of them playing a role in cell migration (Williams et al. 2013).

In contrast to the earlier findings that β-agonist isoproterenol activates ERK, more recent studies using keratinocytes and corneal epithelial cells showed that stimulating β_2-ARs with isoproterenol caused a decrease in the rate of migration due to the inhibition of ERK (Chen, Hoffman, Isseroff 2002; Ghoghawala et al. 2008; Han et al. 2012; Pullar, Chen, Isseroff 2003; Pullar et al. 2006). This inhibition is caused by the activation of PP2A via β_2-AR stimulation, which promotes the dephosphorylation of ERK (Ghoghawala et al. 2008; Pullar, Chen, Isseroff 2003; Pullar et al. 2006). As previously discussed the role of ERK is important in the migratory ability of cells through its role in the activation of proteins like MLCK, calpin, paxillin and FAK (Campbell and Davies 2012; Huang, Jacobson, Schaller 2004; Yu, Deakin, Turner 2010). The results presented in chapter 3 of this thesis were consistent with previous studies in keratinocytes and

corneal epithelia, showing that migration was significantly slowed in airway epithelial cells, vaginal epithelial cells and cervical epithelial cells when the β_2-AR was stimulated with epinephrine, salbutamol, carvedilol or norepinephrine. Interestingly, the effect of epinephrine on the migration rate in airway epithelial cells was dependent on the activity of CFTR. This conclusion was based on the observation that inhibition of CFTR activity with $CFTR_{inh}$-172 or silencing mRNA expression of the channel blocked further reductions in the rate of epithelial restitution following the addition of β-agonists. Moreover, treatment with salbutamol or epinephrine produced nearly identical reductions in lamellipodia area as observed after treatment with $CFTR_{inh}$-172. These findings suggest that at least a portion of the inhibitory response to β_2-AR stimulation in airway epithelial cells is related to a loss of the electrotactic guidance cue that occurs when the wound current is blocked in response to loss of CFTR function. Other mechanisms may also contribute and are discussed below.

One explanation for the reduction in CFTR function associated with carvedilol stimulation is the internalization of the β_2-AR and CFTR upon β_2-AR stimulation. This effect occurs early (within 30 min) in the time course for epithelial restitution and would be expected to cause inhibition of restitution comparable to the effect of a CFTR inhibitor, or partial mRNA silencing and reduced apical membrane expression. However, there is still residual CFTR in the apical membrane, thus it is possible that other mechanisms besides reduced CFTR function contributes to inhibition of migration, especially for epinephrine

which did not reduce the level of CFTR expression in the apical membrane, and as previously mentioned in this thesis the activation of β_2-AR with epinephrine leads to β-arrestin recruitment, which in turn promotes receptor internalization. This internalization would potentially lead to a decrease in CFTR activation due to the decrease in available cAMP which is generated through β_2-AR activation and is essential in the activation of PKA. Previous studies in the keratinocytes and cornea have shown that β_2-AR agonists like epinephrine are able to recruit PP2A, a serine/threonine protein phosphatase through the β-arrestin signaling pathway (Pullar, Chen, Isseroff 2003; Reiter and Lefkowitz 2006). It has also been shown that PP2A and CFTR are found in the same complex (Vastiau et al. 2005) and PP2A dephosphorylates CFTR causing the channel to become deactivated (Thelin et al. 2005; Vastiau et al. 2005). Furthermore recruitment of PP2A dephosphorylates ERK, an important player in cell migration, decreasing its activity (Janssens and Goris 2001; Pullar, Chen, Isseroff 2003), potentially aiding in the decrease in migration seen in airway epithelial cells like what has been shown in keratinocytes and corneal epithelial cells. Recruitment and activation of PP2A may represent an additional mechanism underlying the inhibitory effect of β_2-AR stimulation on airway epithelial cell migration and wound repair.

B. Future directions:

Although the data in this thesis provides new insights on β_2-AR receptor regulation of airway epithelial cell migration and anion secretion, the mechanisms responsible for these effects are not fully understood. To examine the possibility that β_2-AR activation leads to PP2A recruitment and inhibition of CFTR function, experiments could be performed to determine the phosphorylated state of CFTR after treating with a β_2-AR agonists. These experiments would determine if stimulating the β_2-AR with an agonist or bias ligand causes CFTR dephosphorylation over time and if this correlates with changes in ion channel activity. This study would be done by isolating biotinylated proteins from the apical surface of NHBE or Calu-3 cells before and after β-agonist treatment. Total CFTR protein would be identified by western blot analysis and the blot reprobed using phosphoantibodies that target specific phosphorylated states of the channel. This would establish if β_2-AR stimulation decreases the activity of CFTR via dephosphorylation. It is predicted that β_2-agonists cause a decrease in CFTR phosphorylation through the activation of PP2A. The effects of PP2A blockers like okadaic acid or cantharidine, and the effects of PP2A silencing could be used to see if the phosphorylated effects seen with β-agonists are rescued. This would help determine if PP2A is responsible for the dephosphorylation of CFTR which in turn causes a decrease in cell migration. It is suggested that PP2A silenced cells and cells treated with PP2A blocker would have similar concentrations of phosphorylated CFTR protein as the control cells.

Another experiment would be to measure the effects β_2-AR stimulation has on PP2A recruitment to the β_2-AR-CFTR signaling complex in the apical membrane. This could be accomplished by performing a pull-down assay, using antibodies against CFTR to immunoprecipitate the proposed PP2A-CFTR complex before and after β-agonist stimulation. A western blot would then be performed, probing with a PP2A antibody. This could be used to quantitate the relative amount of PP2A associated with CFTR following β_2AR activation and how this corresponds to the phosphorylation state of CFTR. Another experiment would be to examine the effect of PP2A on migration of airway epithelial cells. This could be done by stimulating the cells with epinephrine in the presence of a PP2A inhibitor to see if the inhibitory effect on migration is blocked in absence of functional PP2A. It is suggested that PP2A inhibitor will at least partially rescue the effect epinephrine has on cell migration bringing the migration rate near untreated control cells.

The findings of this thesis help advance the study of obstructive airway disease at the cellular level. These studies provide evidence that common treatments like albuterol or salmeterol have negative effects on the airway epithelium by decreasing CFTR activity and availability, leading to decreased efficiency and ability in wound repair and mucociliary processes. These can cause an increased potential for inflammation and damage to the airways which can lead to exacerbated problems in the later stages of disease. With future studies the ability to identify the mechanism(s) by which these issues occur will

be important in establishing new therapies, drug targets and drug development that are aimed to promote dilation of the airways while preventing CFTR deactivation, and therefore promote mucociliary and repair mechanisms.

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