ABNORMAL NA^+ TRANSPORT BY DISTAL AIRWAY SURFACE EPITHELIA IN CYSTIC FIBROSIS SWINE

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Physiology

University of Saskatchewan

Saskatoon

By

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Abstract

One of the most prevalent hypotheses pertaining to the sequence of events that lead to cystic fibrosis (CF) airway disease is that in normal airway epithelia the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) anion channel inhibits the activity of the Epithelial Na^+ Channel (ENaC). In CF patients, where CFTR is mutated, the inhibition of ENaC is lost. Consequently, ENaC becomes hyperactive, resulting in fluid depletion from the airway, collapse of the mucociliary apparatus, and impaired clearance of microbes, which initiates a cycle of infection and inflammation that may eventually result in respiratory failure. This hypothesis has recently been challenged by reports suggesting that animal models of CF do not exhibit hyperactive ENaC, and that the cilia in their airway appear normally functioning. This constitutes a paradigm shift in CF with serious consequences for current and future CF treatments. However, there is evidence that ENaC hyperactivity in CF may depend on the stimulation of airway epithelia by secretagogues. Thus, using a self-referencing ion selective microelectrode technique with unparalleled spatial resolution, we tested the Na^+ transport properties of the surface epithelia in the distal airway (~2mm) of CFTR-\- swine after stimulation of intracellular cAMP or Ca^{2+} signaling pathways. We show that the epithelial cells located at the folds of the distal airway are capable of both secreting and reabsorbing Na^+, and are sensitive to CFTR and ENaC inhibition with CFTRinh172 and amiloride, suggesting that both ENaC and CFTR are expressed at the folds. Most importantly, using the response to amiloride as an assay for ENaC activity, we detected hyperactive ENaC in forskolin-stimulated CFTR-/- airways. This indicates that CFTR-/- swine airways do indeed suffer from hyperactive ENaC after increased intracellular cAMP, thus potentially collapsing the PCL at those sites.

Acknowledgements

I would like to thank Dr. Juan Ianowski, my thesis supervisor, in this long research process. You fully participated in my academic development and I am especially grateful for your continuous support, encouragement, and trust in me. I would like to thank Dr. Francisco Cayabyab as well, for his constructive and practical advice and for being patient with all of my mistakes. Also, I would like to thank my lab members. You were indispensably helpful and without your support I could not have completed my research. To my parents—you always encouraged me, loved me, and supported my choice in being a researcher. To my wife Mary, for believing in me and supporting me throughout my studying. You patiently endured all my absences from family life during this period of conducting my research and writing my thesis. You gave me the motivation for the completion of this work.

Because of all of you I have achieved another chapter in my life. Thank you.

N.G.

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List of abbreviations

ASL – Airway surface liquid

ATP - Adenosine triphosphate

CaCCs – Calcium-Activated Chloride Channels

cAMP - Cyclic adenosine monophosphate

CF - Cystic fibrosis

CFTR - Cystic Fibrosis Transmembrane conductance Regulator

cGMP – Cyclic guanosine monophosphate

DNA- Deoxyribonucleic acid

ENaC - Epithelial Na^+ Channel

ER - Endoplasmic reticulum

MCC- Mucociliary clearance

MSD - membrane-spanning domains

NBD - nucleotide-binding domains

PCL – Periciliary liquid

PKA - Protein kinase A

PKC - Protein kinase C

SERCA - Sarco/endoplasmic reticulum Ca^{2+}-ATPase

SIET – Scanning Ion-selective Electrode Technique

1. Introduction:

1.1. CF overview

Cystic fibrosis (CF) is one of the most common lethal hereditary diseases among Caucasian populations. In North America, carrier prevalence of the impaired genes is 1:30 while the actual disorder occurs 1 in approximately 3500 live births (Dupuis et al., 2005). Worldwide, approximately 70,000 patients with CF were registered by World Health Organization (World Health Organization, 2002; World Health Organization, 2004). Most CF cases occur in populations of Caucasian descent (Europe, North America, and Australia); however, it does occur in other populations (Cystic Fibrosis Foundation, 2015). According the Canadian CF registry (2014), there are over 4000 CF patients diagnosed in Canada. The current estimated median survival age for Canadians is 58.1 years with a tendency of increasing every year (Canadian CF registry 2018).

Cystic Fibrosis is an autosomal recessive genetic condition caused by mutations (over 2000 mutations) in the gene encoding for the anion channel, Cystic Fibrosis Transmembrane conductance Regulator (CFTR) (Griesenbach, 1999; Boucher, 2003). All epithelial cells that normally express CFTR are dysfunctional in CF patients, resulting in abnormalities in many organs including pancreas, liver, intestine and reproductive organs. However, most of the morbidity and mortality in cystic fibrosis patients arises from CF airway disease (Morton & Glanville 2009; Leitch, & Rodgers, 2013). Cystic fibrosis patients are born with apparently normal lungs. However, in the first few years of life they acquire chronic and unrelenting bacterial infections of the airways (Rajan & Saiman, 2002; Chmiel & Davis, 2003). Patients suffer from transient bronchitis-like airway infections, frequently with the bacterium *Pseudomonas aeruginosa*. Genetic fingerprinting studies show that these first infections are

distinct events; infection develops and is subsequently cleared, and the next bout is caused by a new strain acquired from the environment (Burns et al., 2001). Unfortunately, this stage is temporary, as the airways of nearly all patients eventually become permanently colonized by mucoid *P. aeruginosa* (Nguyen & Singh, 2006), which is a variant phenotype that produces an exopolysaccharide alginate and causes mucoidy, conferring resistance to phagocytosis (Pier et al., 2001) and antibiotics (Hodges & Gordon, 1991). Mucoid *P. aeruginosa* forms a biofilm that cannot be cleared and eradicated (Koch, 2002), and its presence predicts shortened CF patient survival (Henry et al., 1992, Li et al., 2005).

1.2. CFTR

1.2.1. CFTR ,ENaC structures and functions

The CFTR gene consists of nearly 190 kb of genomic DNA found in the long arm of chromosome 7 (7q21-34) (Ellsworth et al., 2000). The product of CFTR gene expression is known to be a multifunctional protein, member of the ATP-binding cassette transporter superfamily that also includes multidrug resistance proteins. These proteins may act as transporters as well as perform regulatory functions modulating transepithelial conductance (Schuetz et al., 1999; Rees et al., 2009, Riordan et al., 1989). A large body of research indicates that in addition to its channel function, CFTR modulates Na^+ reabsorption (Egan et al., 1992; Gabriel et al., 1993; Alton et al., 1993).

CFTR consists of two membrane-spanning domains (MSD1 and 2) followed by 2 nucleotide-binding domains, NBD1-2 (Riordan et al., 1989), and a regulatory domain (R-domain). The R-domain is responsible for gating the channel and it is phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) (Liedtke et al., 2002; Guggino & Stanton, 2006; Auerbach & Liedtke, 2007). The MSDs consists of six membrane-spanning peptides that form

the pore of the channel, connected by hydrophilic loops crucial for maintaining stability of CFTR (Manavalan et al., 1994). Furthermore, specific amino acids encoded in the membrane-spanning domains determine the selectivity of CFTR to Cl^- and other anions (e.g. HCO_3^-, Sheppard & Welsh, 1999). The NBDs regulate binding and hydrolysis of ATP, providing the channel with the energy needed for opening and closing the pore (Anderson et al., 1991; Gadsby et al., 1999). Several studies have shown that CFTR R-domain comprises multiple protein kinase-A phosphorylation sites that are important for gating activation (Chappe et al., 2005; Hwang et al., 2013). Phosphorylated R-domain initiates binding and hydrolysis of ATP by NBDs leading to changing of pore conformation (open state) (Sheppard, & Welsh, 1999).

All conductive airway epithelia are able to reabsorb sodium through the amiloride-sensitive apical ENaC (Mall et al., 1998; Kunzelmann et al., 2002; Scott et al., 1987; Bange et al., 2008). ENaC consists of three homologous α-, β-, and γ-subunits (Canessa et al. 1994). Proper function of the ENac channel requires polymerization of all three subunits in the cell membrane (Staruschenko et al., 2005; Stockand et al., 2008). Subunits include two intracellular segments with NH2- and COOH- terminals separated with two transmembrane (TM, TM2) sequences and one extracellular domain (Canessa et al., 1993). Regulation of ENaC activity depends on structure and composition of intracellular terminals of βγ-subunits. Some studies showed elevated ENaC activity together with increased expression of the channel due to mutations of C- and N-termini of the βγ-subunits (Firsov et al., 1996; Tamura et al., 1996). As in many other channels, modulation of ENaC activity could be facilitated by changing two main parameters: quantity of expression and open probability (Garty & Palmer 1997; Rossier 2002).

1.2.2. CFTR mutations.

In 1946, Anderson and Hodges first found evidence that supported the genetic nature of cystic fibrosis (Anderson & Hodges, 1946). They estimated an approximately 25% rate of occurrence of CF in afflicted families, which is explained by the recessive type of CF inheritance. Further investigation on CF patient abnormalities revealed an electrolyte imbalance of Cl^- in sweat secretion that enlightened the etiology of CF (Majka et al., 2003). Since the CFTR gene was identified in 1989 (Kerem et al., 1989; Riordan et al., 1989), the biological basis of the ion transport abnormalities became clearer. Over the last three decades more than 2000 CF causing mutations of CFTR were discovered (Castellani et al., 2008; http://www.genet.sickkids.on.ca/StatisticsPage.html). CFTR mutations are grouped in six classes depending on the type of dysfunction they cause. Class I includes mutations encoding nonsense mRNA, which cannot be translated. Class II mutations cause protein misfolding that precludes CFTR trafficking to the membrane. Thus, expression of CFTR channels in the cell membrane is minimized. The most common mutations found in 70% of CF patients are class II mutations. A deletion of a phenylalanine in position 508 of MSD1 (Phe508del) is one of the common second class mutation that leads to misfolding of the channel (Bobadilla et al., 2002). Class III mutation is a "gating" type of mutations that leads to the production of dysfunctional proteins. CFTR proteins are synthesized and trafficked to the cell membrane, but the opening of the channel is disrupted due to inability to bind and to hydrolyze ATP (Logan et al., 1994; Bompadre et al., 2007). Common Class III mutations are G551D and G1349D mutations in NBD1 and NBD2 respectively, that diminish the ability to bind and hydrolyze ATP , thus reducing gating by 10 to 100 times (Bompadre et al., 2007). G551D is the third most common mutation that causes severe CF symptoms (http://www.genet.sickkids.on.ca/cftr/app). Class IV mutations are those that result in reduced anion conductivity. These are associated with a moderate form of CF lung disease that

usually manifest in early age (O'Sullivan et al., 2006). One of the most frequent mutations of the class is R117H, where an arginine is substituted with histidine at position 117 of CFTR. Some of the mutated proteins are incapable of folding and undergo degradation upon synthesis. Those CFTRs that are properly folded form channels with by approximately 25% reduced in ion conductance, as well as lower open probability (Yu et al., 2016). In class V mutation, altered stability of mRNA produces damaged proteins and the expression of normally functioning CFTRs is reduced; thus, overall ion conductance is reduced compared to healthy epithelium, causing non-symptomatic or mild clinical phenotype (De Boeck et al., 2014). For instance, the splicing mutation 2789 + 5 G-A (frameshift splice) producing only 4% of correctly spliced CFTR, while the rest results in a truncated CFTR (Highsmith et al., 1997). Class VI mutations generate unstable proteins or proteins with impaired anchoring segment and increase turnover of the protein (Wang et al., 2014; Pettit & Fellner, 2014). The C-terminal truncation of CFTR due to premature activation of a stop codon or frameshift results in significant pancreatic deficiency and mild lung disease (http://www.genet.sickkids.on.ca/cftr/).

CFTR mutations affect the function of epithelial tissues, which normally express CFTR, altering the function of many organ systems, such as the respiratory system and the gastrointestinal tract. Currently, most of the mortality and morbidity faced by CF patients results from lung disease.

1.3. CFTR and epithelial tissues.

1.3.1. Epithelial tissue structure and function.

Of all the tissue types found in the animal body, the epithelial tissue (from the Greek *epi-*

over and *thele*-peels) is the most ancient, which in the phylo- and ontogenesis arise first (Kenney et al., 2015). The epithelium is a layer of epithelial cells attached by tight joints to form a sheet of tissue (Fox, 2006). Thus, separating the external from internal environment, as well as lining all internal organs, and forming glandular organs in animals (Kenney et al., 2015).

Epithelia separate the body from the environment and perform important functions to protect the underlying tissues from various external insults, such as chemical, mechanical, and infectious challenges (Griesenbach, 1999; Boucher, 2003). Epithelial tissues also mediate the exchange of solutes and water with the environment by carrying out reabsorption and secretion of substances (Sherwood, 2015). For example, through the intestinal epithelium, products of food digestion are absorbed into the blood and lymph. In addition, the epithelium covering the internal organs creates compartments with conditions required to support specialized cells (e.g. neurons) to perform their function. Finally, glandular epithelia have a secretory function, synthesizing and secreting diverse substances required for different processes. For example, secretions from the pancreas facilitate digestion of proteins, fats and carbohydrates in the small intestine, or airway submucosal glands that are involved in innate immune defense (Stanfield, 2012).

1.3.2. Epithelia in the airway and cystic fibrosis disease.

Epithelial lining is present in the entire bronchial tree starting from the trachea and continuing through the airways toward terminal bronchioles and alveoli (Ayers & Jeffery, 1982). Large bronchi are lined with ciliated pseudostratified columnar epithelium that varies in thickness depending on bronchial division and is gradually decreasing in height. In terminal bronchioles the epithelium becomes flatter, turning in cuboidal form (Jeffery, 1983; Jeffery, 1995). Ciliated epithelial cells are the major component of the respiratory epithelium and are found through all conductive airways. The synchronised motion of the cilia drives the movement

of the mucus layer, and pathogens trapped within it, towards the pharynx and out of the airways. This process, termed mucociliary clearance (MCC), and is central for airway innate defense, and provides the mechanical removal of pathogens and inhaled particles from lungs. In cystic fibrosis, mucociliary clearance fails, thus facilitating infection and inflammation that may eventually lead to respiratory failure (Griesenbach, 1999; Boucher, 2003).

1.4. Mucociliary clearance.

Mucociliary clearance is a complex mechanism of lung innate defense mechanism that helps eradicate microorganisms and foreign particles from the airways (Robinson et al., 2000; Knowles & Boucher, 2002; Sagel et al., 2011). Airway epithelial cells in the respiratory tract contribute to the production of mucus, and the cilia create a mechanical force for MCC (Wanner et al., 1996).

Mucus is a gel-like secretion, overlaying the ciliated epithelia (Thornton & Sheehan, 2004; Cone, 2009). Mucus gel consists of the 97% water and 3% solids, including mucins and antibacterial peptides. Mucins are large glycoproteins (~3×10^6 D) that consist of one or several protein monomers with abundant regions of serine and threonine residues bound to sugar monomer O-glycan (Thornton & Sheehan, 2004). Approximately 30% of all solids in mucus are mucins (Thornton et al., 2008). The human genome contains 17 genes encoding mucins, among them 10 are membrane-bound and the rest are secreted (Hattrup & Gendler, 2008). Some mucins have terminal cysteine-rich domains allowing them to polymerize due to disulfide bonds. Such polymerization forms the mesh of mucins that contribute to the gel-like properties of mucus (Thornton & Sheehan, 2004). In addition to mucins, respiratory mucus contains antimicrobial peptides, such as siderocalin, which binds to bacterial siderophores depriving the microorganism from iron as well as modulating pulmonary macrophages, converting them to anti-inflammatory

phenotype from pro-inflammatory one (Warszawska et al., 2013). Other antimicrobial compounds include lactoferrin, lysozyme (Bowes et al., 1981; Hinnrasky et al. 1990), lactoperoxidase, which facilitates production of the antimicrobial hypothiocyanite (Gerson et al., 2000; Wijkstrom-Frei et al., 2003), cathelicidin, and β-defensins that kill bacteria by destroying bacterial membranes (Bals et al., 1998).

The second component of MCC, in addition to mucus, is cilia movement, since ciliary beating drives mucociliary clearance (Knowles & Boucher, 2002). The velocity of mucociliary clearance depends, among other factors, on the ability of the cilia to move efficiently. In CF lungs cilia movement seems to be compromised due to the dehydration of airway surface liquid, reducing the volume of airway surface liquid around the cilia, thus resulting in the collapse of the cilia, abrogating ciliary movement (Tarran et al., 2006; Rubin, 2007).

1.4.1. Airway surface liquid

The layer of mucus discussed above is not in direct contact with the surface epithelia. Instead, there is a layer of fluid between the mucus layer and the epithelial cells termed the periciliary liquid (PCL) layer. Together, the mucus layer and the PCL form the Airway Surface Liquid (ASL).

The PCL layer is regulated at approximately 7 um in height, ideal to facilitate cilia movement that drives mucociliary clearance of inhaled particles, including pathogens. In distal airways, the PCL is regulated by airway surface epithelial cells though the active secretion and reabsorption of ions and water (Widdicombe, 2002).

The regulation of PCL volume is mediated by two competing activities: Na^+-driven fluid reabsorption versus Cl^--driven fluid secretion into the lumen of the airway. Reabsorption of PCL

involves activation of apical epithelial Na^+ channels (ENaC) and basolateral N^+/K^+- ATPase. In contrast, PCL secretion requires activation of CFTR on the apical membrane and $Na^+/K^+/2CL^-$ cotransporters facilitates Cl^- uptake on basolateral membrane, providing transcellular transport of chloride ions (Frizzell et al., 1981). The failure to regulate of PCL volume in CF has long been considered the pathogenic event in lung disease.

1.5. Pathogenesis of CF

For more than 20 years the consensus in the CF field was that in normal airways CFTR inhibits ENaC function and that this inhibition is lost in CF airways (Boucher et al., 1986, Briel et, al., 1998), resulting in hyperactivity of ENaC, which depletes the PCL, collapsing the cilia, and eventually abrogating mucociliary clearance in the airway (Hobbs et al., 2013). This inability to clear bacteria would initiate a pathological cascade in CF lungs, promoting infection, inflammation, and airway remodeling (Boucher, 2007).

This long-standing and widely accepted hypothesis has recently been challenged by studies utilizing new CF animal model systems. Welsh and coworkers reported that the upper airways of CFTR-\- pigs do not display hyperactive ENaC or increased Na^+ reabsorption from the PCL, the cilia and PCL appear normal (Chen et al 2010), and the basal mucociliary clearance is normal (Hoegger, 2014). Other studies in CFTR-/- ferrets (Sun et al., 2010, Fisher et al., 2013) and rats (Tuggle at al., 2014) as well as in human CF cell cultures (Itani et al., 2011) found no evidence of hyperactive ENaC. Moreover, Quinton and co-workers suggested that ENaC and CFTR are not expressed by the same cells of the airway surface epithelia (Shamsuddin, & Quinton, 2012; Flores-Delgado et al., 2016), contradicting previous results (Grubb et al., 2012). Instead, they propose that cells located in the pleats of the distal airway epithelia express CFTR

and specialize in secreting fluid (Fig. 1A), while cells on the folds express ENaC and specialize in reabsorption (Shamsuddin & Quinton, 2012; Flores-Delgado et al., 2016), thus, ENaC-CFTR interaction is not possible.

1.6 Rationale

These reports constitute a paradigm shift in our understanding of CF airway disease pathogenesis and challenge the very basis of some current CF treatments, such as nebulization with hypertonic saline, and the development of new ones based on ENaC activity modulation (Goralski et al., 2010). However, the contribution of hyperactive ENaC to CF pathogenesis has not been fully tested since the phenotype displayed by CF airway epithelia may depend on the stimulation of the epithelia cells by secretagogues. For example, CFTR-/- swine airway display abnormal mucociliary clearance only after cholinergic stimulation of the airway (Hoegger et al., 2014), and stimulation of intracellular cAMP increases ENaC hyperactivity in CF airway epithelial cells (Boucher et al., 1986; Stutts et al., 1995; Chambers et al., 2005). The activity of ENaC in CFTR-/- swine after stimulation of epithelial cells treated with secretagogues has never been tested.

1.7 Objectives

Therefore, we investigated ion transport properties of the distal airways (~2 mm diameter) in wild-type and CFTR-\- swine after stimulating intracellular cAMP or Ca^{2+} signaling with forskolin or thapsigargin, respectively. We used a high resolution self-referencing ion-

selective microelectrode technique that allows us to measure Na^+ transport properties at the folds of the distal airway.

1.8 Hypotheses:

We propose two main hypotheses:

1) Airway surface epithelial cells can both secrete and reabsorb fluid and ions (Fig. 1-1)

2) In CF airways, Na^+ transport is hyperactive

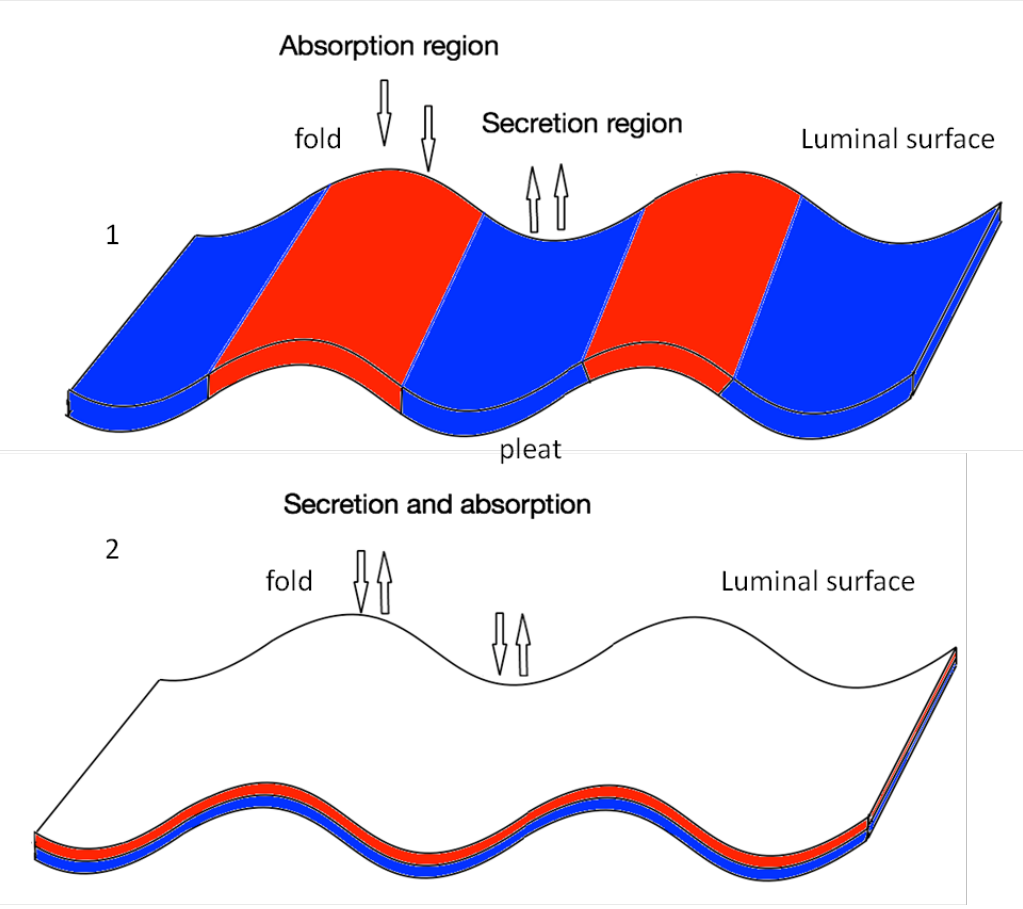

Figure 1-1: Schematic illustration of two alternative theories of ASL regulation in distal airways. 1) The distal airway consists of two cell populations with different transport properties. The epithelial cells in the pleats secrete liquid, Na^+ and Cl^- in to the lumen via CFTR-mediated transport. The folds reabsorb fluid, Na^+ and Cl^- though an ENaC process. 2) A small airway epithelium is a homogeneous cell population. Cells in fold and pleat regions are capable of bidirectional sodium transport.

2. Methods

2.1. Animals.

We used lungs from 2-7 days old gut-corrected CFTR knockout pigs (*CFTR*$^{-/-}$*;TgFABP>pCFTR* pigs) purchased from Exemplar Genetics (Exemplar Genetics, Sioux Center, IA). Lungs from male and female ~7 days old wild-type pigs were used as controls (Prairie Swine Centre, University of Saskatchewan). The CFTR-/- and wild-type lungs were dissected within 15-30 min of euthanasia, clamped to prevent fluid penetration into the lumen, and placed in ice-cold Krebs-Ringer solution (mmol/l: 115 NaCl, 2.4 K_2HPO_4, 0.4 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 10 glucose at pH = 7.4) containing 1 μM indomethacin to minimize tissue exposure to endogenously generated prostaglandins and equilibrated with 95% O_2-5% CO_2. We used the tissue for experimentation within 12 h of euthanasia since preliminary data show no significant decay in the viability of the tissue within that time (see Appendix I). All procedures were approved by the Animal Care Committee of the University of Saskatchewan.

2.1.1. Tissue preparation

Blocks of tissue (3 cm^3) were excised from the lower lobes of the lungs. Using a dissecting microscope we identified and dissected small airways (~2 mm in diameter and ~5 mm in length). The airway preparation was cut longitudinally to expose the lumen and producing a block of tissue of ~6 by 5 mm, and ~1 mm height. Prior to and during ion flux measurements the airway tissues were maintained at 37 ± 2°C using a custom-built temperature control system. We tested the ion transport properties of the folds (i.e. regions that extend into the lumen of the airway) since it provides easier access for the electrodes.

2.2. Scanning Ion-selective Electrode Technique (SIET).

Epithelial solute transport generates a concentration gradient of the transported solute adjacent to the cell membrane, i.e. unstirred layer (Fig. 2-1A). The intensity of the unstirred layer is proportional to the epithelial transport rate (Barry and Diamon 1984). Hence it is possible to map and measure patterns of solute flow, in our case Na^+, across an epithelial surface by measuring the ion concentration gradients in the unstirred layer and using Fick's law of diffusion to calculate the diffusion rate, which in steady state matches epithelial Na^+ flux (Kfihtreiber and Jaffe, 1990).

We used SIET (Fig 2-1 A and B, Applicable Electronics, Forestdale, MA, USA), a self-referencing non-invasive method, to measure transepithelial Na^+ flux with great spatial and temporal resolutions (Smith et al., 1994, Piñeros et al., 1998, Somiesk and Nagel 2001). Na^+ flux measurement is a surrogate for NaCl (or $NaHCO_3^-$) transport across the epithelia since Na^+ transport rate must be matched by a counterion (e.g. Cl^- or HCO_3^-) to maintain electroneutrality.

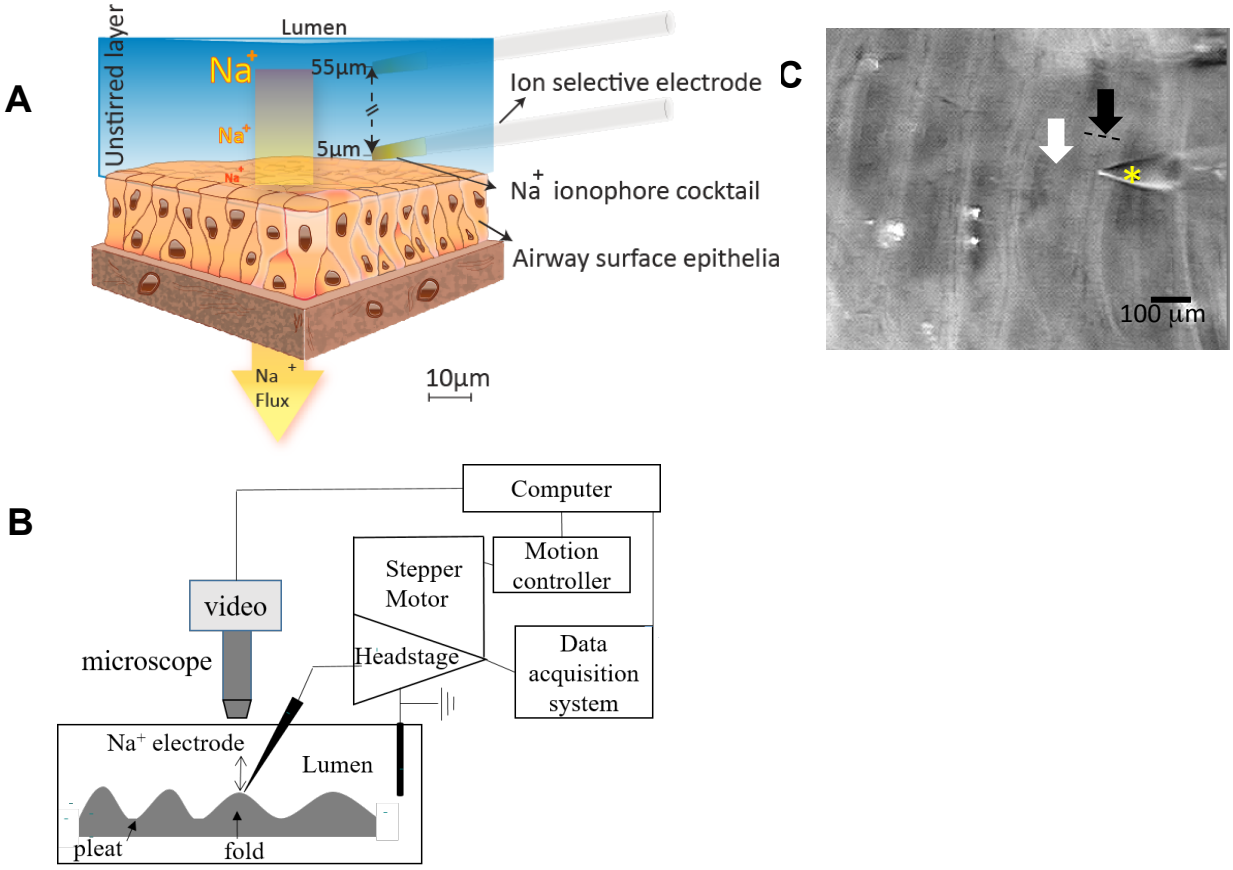

Figure 2-1: SIET experimental setup. A) Schematic diagram of the Na^+ gradient (background colour intensity) generated by an epithelium that is transporting Na^+ from the apical to basolateral side (red arrow) which generates an unstirred layer. SIET uses Na^+-selective microelectrode to measure the Na^+ concentration gradient and calculate the Na^+ flux across the epithelia. B) Schematic representation of the SIET device. A video camera connected to a microscope facilitates placement of the electrode in the desired location. Movement of the electrode is computer-controlled. Measurements are collected and processed by the computer. C) Sample image of small airway. Preparations are placed luminal (i.e. apical) surface up in a temperature-controlled chamber and bathed in Krebs-Ringer solution. The tip of the Na^+ microelectrode (*) is placed on the folds (black arrow) of epithelium which appear as light-grey to white ridges as opposed to the pleats (white arrow).

SIET Na^+ flux measurements are accomplished by conducting a series of computer-controlled movements of a Na^+-selective microelectrode to measure Na^+ concentration at two points within the unstirred layer, which preliminary experiments determined needed to be separated by 50μm (Fig. 2-1 A and C). Ion selective microelectrode voltage was recorded for a sampling period of 0.5s. Then, the ion selective microelectrode was moved 50 μm away, followed by a 4s wait period, to ensure the re-establishment of the ion unstirred layer that may have been disturbed by the microelectrode movement, before another 0.5s sampling period (O'Donnell & Ruiz-Sanchez 2015). A whole measurement cycle was completed in ~9.5 s. Reference measurements were collected at locations at least 0.5 mm away from the specimen, where no unstirred layer is detectable. All measurements made on the specimen were referred to the reference readings taken periodically during the experiment.

The Na^+-selective microelectrode voltage gradient was converted to concentration gradient using:

$$\Delta C = \mathrm{Cb} \times 10^{(\Delta V/S)} - \mathrm{Cb}$$

where ΔC corresponds to Na^+ concentration gradient (μmol cm^{-3}); Cb is background Na^+ ion concentration (μmol cm^{-3}); ΔV is the voltage gradient (mV), and S represents the microelectrode slope (mV) (Donini and O'Donnell, 2005).

Using the Fick's law of diffusion we converted the concentration gradients into ion fluxes (Smith et al., 1994):

$$J = D\Delta C \,/\, \Delta X$$

where J is Na^+ ion flux across the epithelial cells measured in pmol $cm^{-2}\ s^{-1}$; D is diffusion coefficient for the sodium ion ($1.55\times10^{-5}\ cm^2\ s^{-1}$); ΔC is concentration gradient, ΔX is the distance in cm between the two measuring points.

2.2.1. Na^+–selective microelectrodes construction

Na^+–selective microelectrodes were constructed from borosilicate capillaries (TW150-4; WPI, Sarasota, FL, USA). Electrodes were pulled using a Narishige PC-100 puller, and salinized with dichlorodimethylsilane at 250°C for at least 6 hours (Doninni and O'Donnell, 2005). The tip of each electrode was filled with a 50 μm column of Na^+ Ionophore II Cocktail A (Fluka) and backfilled with 100 mmol l^{-1} NaCl solution. The microelectrodes were calibrated in 150 mmol l^{-1} NaCl and 15 mmol l^{-1} NaCl + 135 LiCl solutions. Electrodes that display a voltage change smaller than 50 mV per 10 times change in Na^+ concentration in the calibration solutions (i.e. slope of the electrode) were excluded from the experiment. Our electrodes had an average slope of 55±5 mV (n=78). The reference electrodes were 4% agar bridges in 500 mmol l^{-1} KCl.

2.3. Reagents

CFTRinh-172 was obtained from Cedarlane Labs (Burlington, ON, CA). Forskolin and thapsigargin were purchased from Sigma-Aldrich. Stock solutions of CFTRinh-172, forskolin, and thapsigargin were dissolved in DMSO. The final concentration of DMSO was less than 0.1%.

2.4. Statistics

Data are presented as mean ± S.E.M. The data sets that met the assumption of normality and homogeneity of variances where analyzed with parametric tests, ANOVA or Student's t test. For those that did not, we performed non-parametric tests using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, US), and $p < 0.05$ was considered significant. Preliminary

experiments showed that a sample size of 6 samples was sufficient to ensure adequate power to detect the effects of treatment on PCL secretion. We did not exclude any data from our analysis.

3 Results

3.1 SIET measurements in distal airway epithelium

3.1.1 SIET electrode efficiency

To determine the how well our SIET set up and sampling protocols measured ion flux we tested the efficiency of SIET Na^+ flux measurement (Piñeros et al., 1998). We produced an artificial Na^+ source by filling a micropipette (tip diameter ~10μm) with 1M NaCl plus 0.5% (w/v) agar to minimize bulk water flow. The source was placed 50μm away the bottom of the experimental chamber filled with Krebs-Ringer solution containing 140 mM Na^+ (Piñeros et al., 1998; Donini, & O'Donnell, 2005).

Theoretical values for the Na^+ gradient generated by the source pipette were calculated according to Piñeros et al., 1998 (Fig. 3-1 A).

$$\Delta V=S[(-U\ \Delta r)/(C_B\ r^2 + U\ r)]/\ 2.3$$

where ΔV is the change (mV) over the electrode excursion, S is the slope of the electrode, r is the distance from the source, Δr is the amplitude of vibration, C_B is the background concentration of Na^+ and U is an empirical constant. To calculate U we measure Na^+ electrode voltage at known distances from the Na^+ source and calculated Na^+ concentrations values using the calibration curve and plotted them vs the inverse of the distance from the Na^+ source ($1/r$) adjusted the data to the equation (Fig. 3-1 B, Piñeros et al., 1998):

$$C = C_B + U/r$$

To determine the efficiency of our SIET Na^+ microelectrode we compared the theoretical value to the actual Na^+ flux measured using the SIET with the Na^+ microelectrodes and protocols

used in our experiments (Fig 3-1 A). The efficiency of SIET measurements is calculated as the ratio of the slope of the experimental data to the slope of the theoretical data (Fig. 3-1 A, Piñeros et al., 1998). The efficiency of our SIET Na^+ flux measurement was 75%, which is consistent with previous reports (Fig. 3-1 A, Rheault and O'Donnell, 2001).

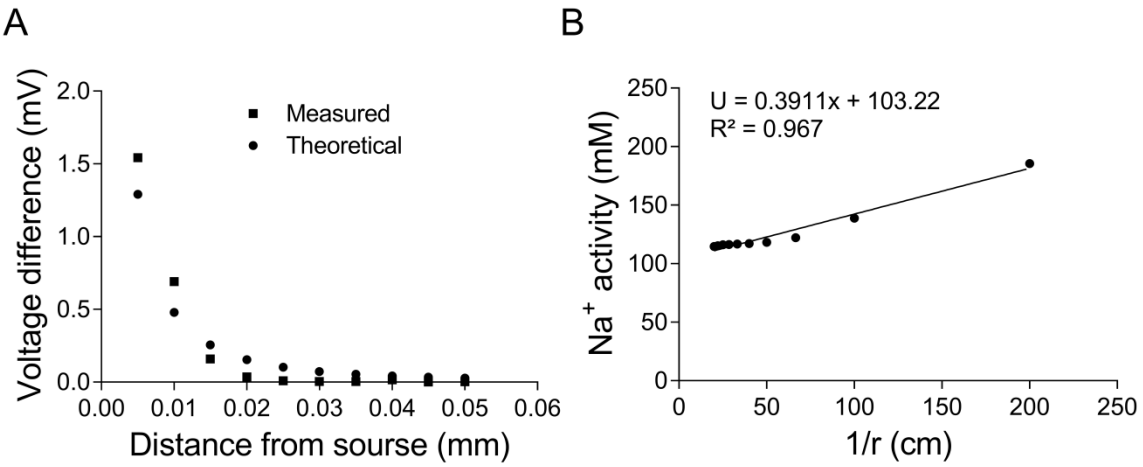

Figure 3-1: Electrode efficiency. A) Na^+ microelectrode voltage difference as a function of distance from a Na^+ source. Experimental measurements (circles) were made by vibrating the microelectrode through an amplitude of 100μm at each distance while the theoretical values (squares) where calculated. B) To calculate the empirical constant *U*, static measurements were made at a series of distances from the source, and the millivolt outputs were then converted to activity values and plotted versus the inverse of the distance from the Na^+ source. Using the method of Piñeros et al. 1998, electrode efficiency (75%) was calculated as the ratio of the slope of the experimental data to the slope of the theoretical data.

3.1.2 SIET spatial resolution

We estimated the spatial resolution of our SIET experimental protocol by measuring the ability of SIET to resolve two Na^+ point sources (Fig. 3-2 A, Somiesk and Nagel 2001). The point sources consisted of two borosilicate micropipettes filled with 1M NaCl in 0.5% agarose, with ~5µm tip diameter and separated by 35 µm. Using SIET we measure the Na^+ flux at each point source and the space separating the sources every 5 µm (Fig. 3-2 B). The data shows that SIET can easily resolve these two point sources, suggesting that the space resolution of the technique allow us to resolve fold from pleat, which in our preparation are separate by ~50µm. In addition, we determine whether the Na^+ gradient measured at the folds was influenced by the ion transport across the pleat. We measured the forskolin-stimulated Na^+ flux at the fold and on the same plane but over the pleats, located ~50 µm away (Fig. 3-3 A). The results show that over the pleats, forskolin produced a very small Na^+ gradient, which is only ~0.02 % of the flux measured on the folds (Fig. 3-3 B and C). These measurements indicate that any contribution of the pleat to our Na^+ flux measurements on the fold is insignificant.

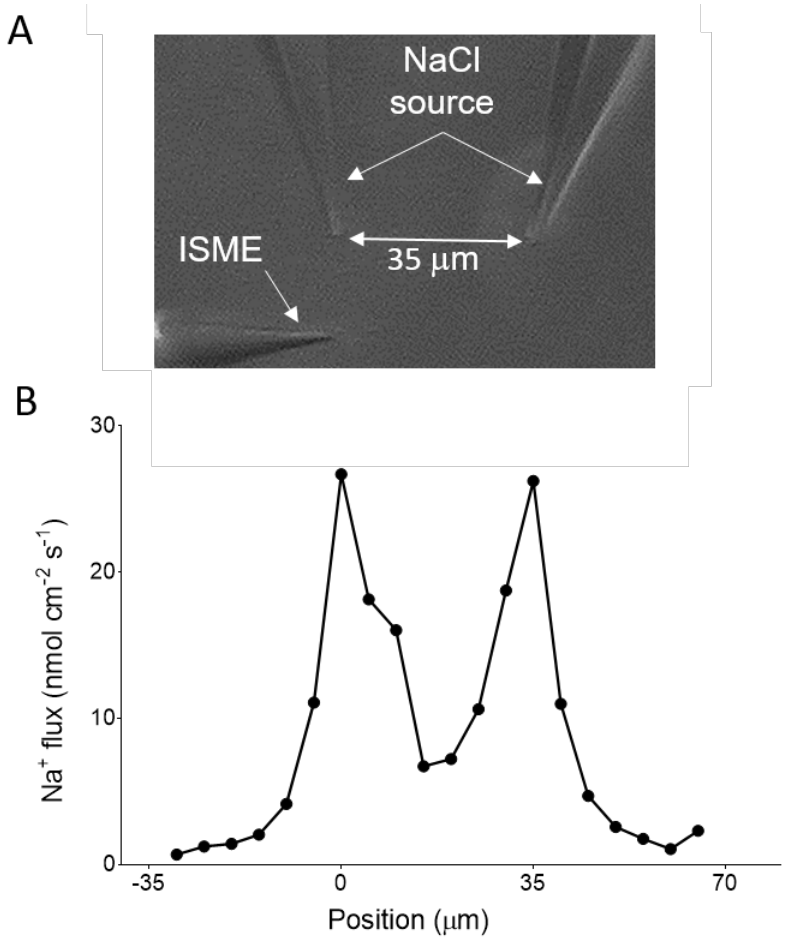

Figure 3-2: SIET spatial resolution. We estimated the spatial resolution of our SIET experimental protocol by measuring the ability to resolve two Na^+ point sources. A) Image of an experimental set up depicting the two borosilicate micropipettes filled with 1M NaCl in 0.5% agarose, with ~5µm tip diameter and separated by 35 µm. Using the Na^+ selective microelectrode (ISME), the Na^+ flux was measured at each point source and the space separating the sources every 5 µm. B) The data shows that SIET can easily resolve these two point sources, suggesting that the space resolution of the technique allow us to resolve fold from pleat in our preparation.

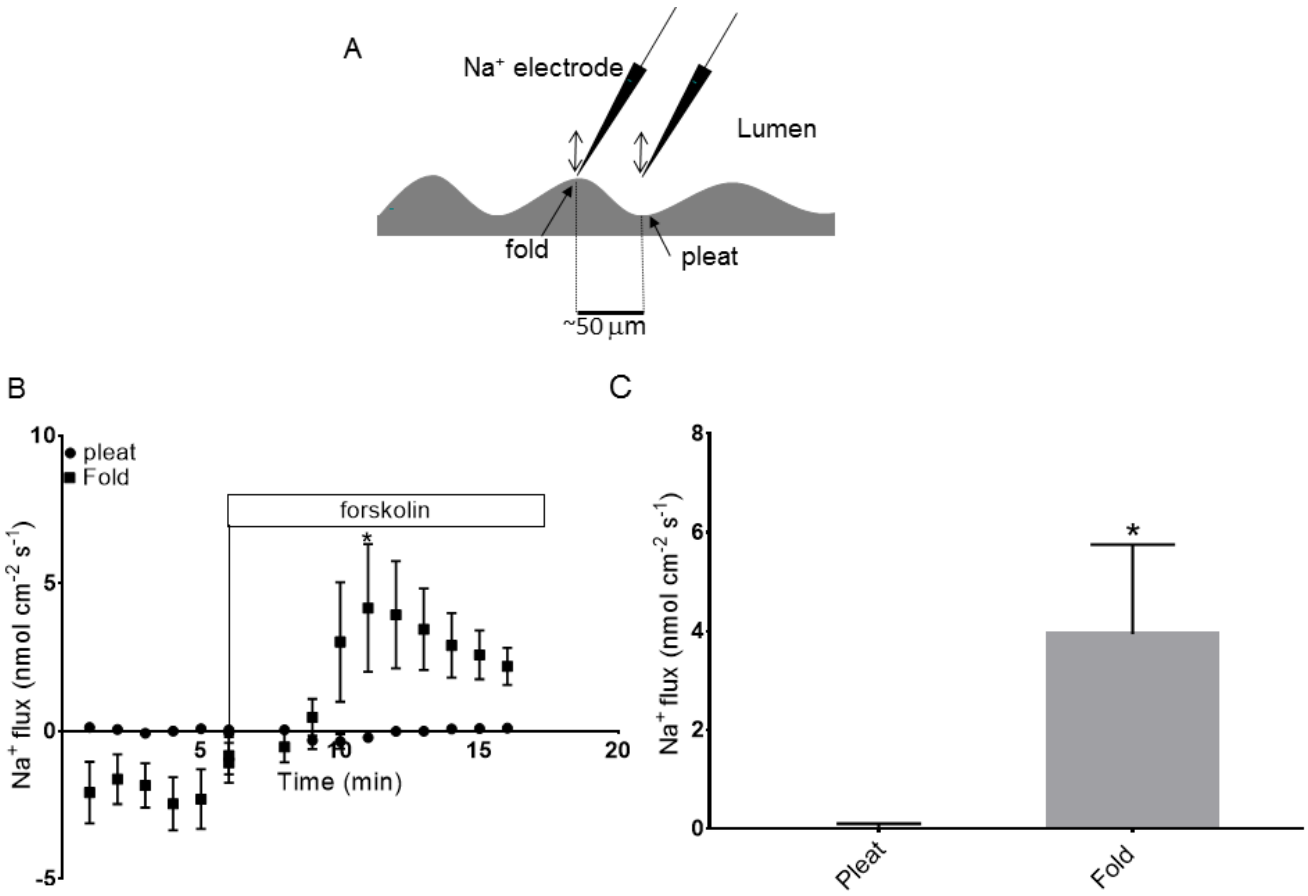

Figure 3-3: Contribution of pleat Na^+ transport to fold measurements. A) In order to determine whether the Na^+ gradient generated by the pleat contributes to our measurements at the folds, we measured forskolin-stimulated Na^+ flux at the fold and on the same plane but ~50 µm to the side, where the pleats are located. B and C) The results show that the Na^+ flux was 3.9 nmol cm^{-2} s^{-1} at the folds and 0.001 nmol cm^{-2} s^{-1} over the pleats which only ~0.02% of the forskolin signal measured at the folds. Asterisk denotes statistical significant difference.

3.2 Evaluation of the ion transport properties of the distal airway surface epithelium

Welsh and coworkers used the response to amiloride, an ENaC blocker, as an assay for ENaC activity in the upper airway of CFTR-/- swine (Chen et al., 2010) and CF human epithelial cell cultures (Itani et al 2011). We followed a similar approach to test for ENaC activity in the distal airway of wild-type and CFTR-\- swine. We measured Na^+ flux across the folds, i.e. the segments of tissue protruding into the lumen of the airway, of the distal airway epithelia (~2 mm diameter) using Scanning Ion-selective Electrode Technique (SIET) that is capable of spatial resolution unparalleled by previous studies in CF. We detected a basal level of Na^+ flux in both wild-type and CFTR-/- swine with a significantly different frequency distribution (Fig. 3-4). These results show that the folds are capable of both secreting *and* reabsorbing fluid, contradicting the hypothesis that the folds of the tissue are specialized for reabsorption (Shamsuddin, & Quinton, 2012; Flores-Delgado et al., 2016).

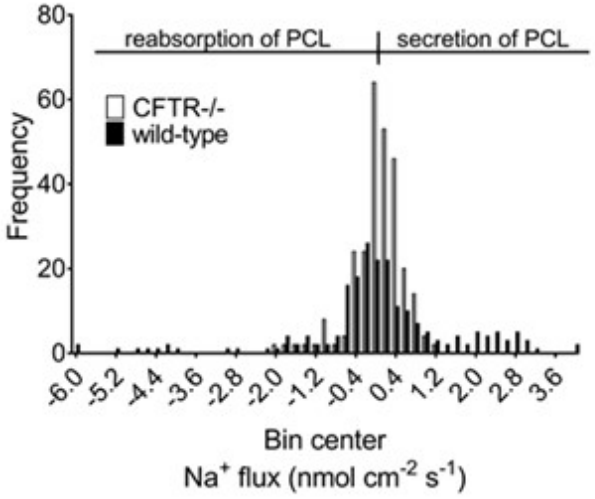

Figure 3-4: Frequency distribution of basal Na^+ flux in wild-type and CFTR-/- distal airway. The frequency distribution of wild-type and CFTR-/- swine is statistically different, with the data more tightly grouped around 0 values for CFTR-/- animals ($p < 0.05$ Chi-square test). The mean Na^+ flux of -0.3 ± 0.1 in wild-type, and 0.05 ± 0.03 nmol cm^{-2} s^{-1} in CFTR-\- ($p < 0.05$ Unpaired t test with Welch's correction, wild-type n = 216 from 12 pigs, CFTR-/- n = 269 from 10 pigs).

Since cAMP has been shown to upregulate ENaC activity and increase Na^+ reabsorption in CF but not in wild-type airway cell cultures (Boucher et al., 1986; Stutts et al., 1995, Chambers et al 2007), we tested the effect of amiloride on preparations pre-stimulated with forskolin, an adenylate cyclase activator, to increase intracellular cAMP. Our results show that stimulation of wild-type preparations with forskolin (1μM) triggered a transient increase in Na^+ secretion into the lumen of the airway that peaked 4 ± 1 min after stimulation at 5 ± 2 nmol cm^{-2} s^{-1}, followed by a decrease in Na^+ reabsorption to an average of 0.01 ± 0.7 nmol cm^{-2} s^{-1} (Fig. 3-5 A, n = 15 sites from 8 pigs). Some sites responded to forskolin by triggering Na^+ secretion initially and later reabsorption, demonstrating that a single site in the folds is able to both secrete and reabsorb fluid, rejecting Quinton and coworkers' hypothesis (Fig. 3-5 A insert, Shamsuddin and Quinton, 2012; Flores-Delgado et al., 2016). The effect of forskolin was blocked by treating wild-type preparations with the CFTR blocker, CFTRinh172, (100 μM, Fig 7B, n = 21 sites from 9 pigs). In CFTR-/- swine, forskolin did not significantly increase Na^+ secretion (Fig. 3-5 C and D, n = 13 sites from 10 pigs, $p<0.05$ Kruskal-Wallis test, Dunn's multiple comparison). These results are consistent with reports in human (Itani et al., 2011; Joo et al., 2002), CFTR-/- swine (Chen et al., 2010), rat (Tuggle et al., 2014), and mice preparations (Grubb et al., 1994; Boucher 2007) suggesting that cAMP-stimulated fluid transport requires CFTR and is missing in CF airways.

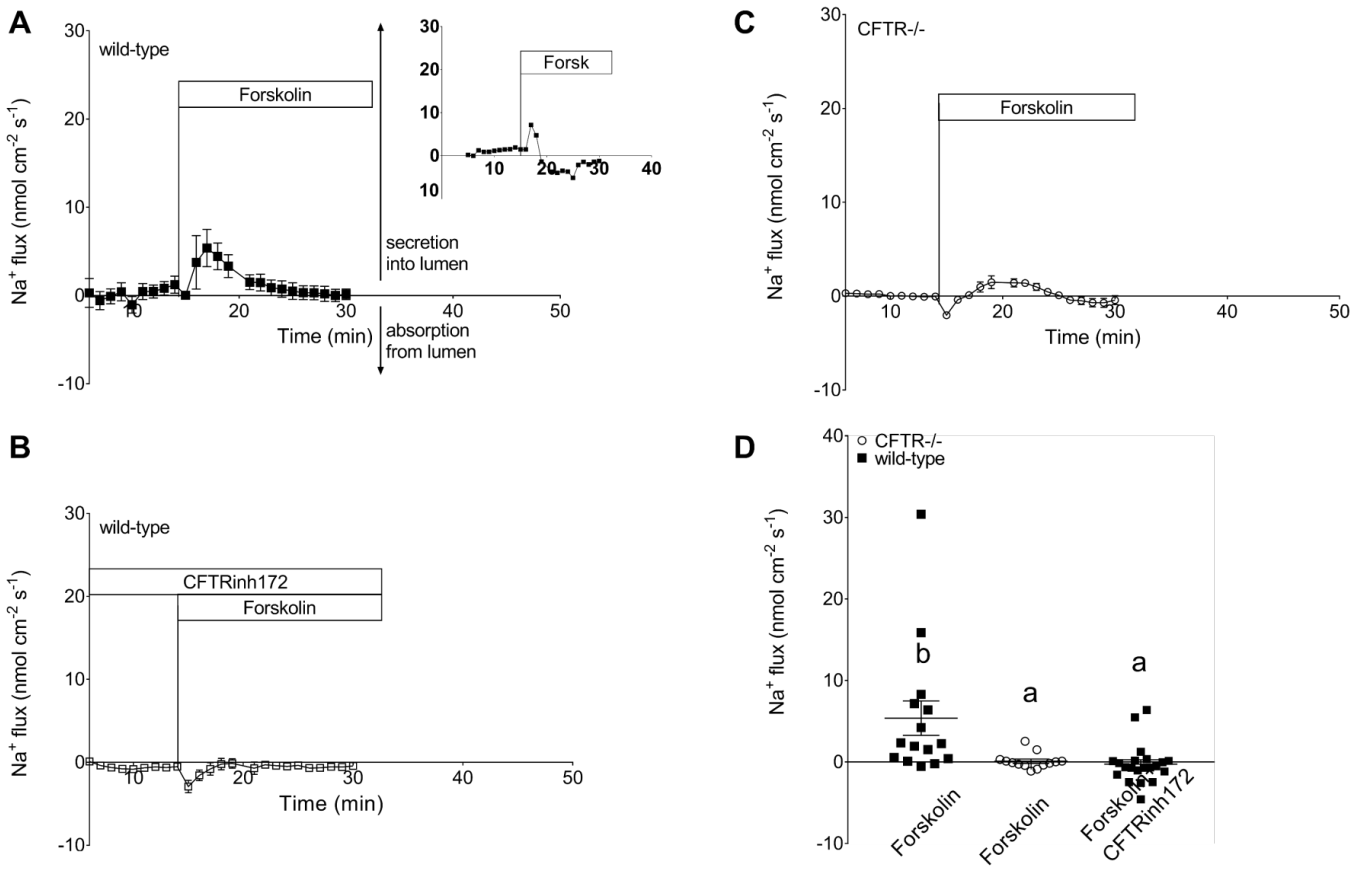

Figure 3-5: Effects of foskolin on WT and CFTR-/- small airway epithelia. Positive values indicate Na^+ flux from the basolateral side into the lumen of the airway, and negative values indicate transport in the opposite direction. A) Wild-type response to forskolin stimulation (1μM). Insert represent a sample trace showing Na^+ secretion and reabsorption by the same site. B) Wild-type preparations incubated with the CFTR blocker, CFTRinh172 (100 μM) and C) CFTR-/- airway. D) Peak response to forskolin for wild-type swine was significantly larger than the response of preparation incubated with the CFTR inhibitor, CFTRinh172, and that displayed by CFTR-/- swine airway, columns labeled with different letters are statistically significance.

Pre-incubating preparations with forskolin and then treating them with amiloride produced an increase in Na^+ secretion into the PCL, consistent with blockage of ENaC. The effect of amiloride was significantly larger in CFTR-/- than in wild-type swine (Fig 3-6A, CFTR-/- n = 12 sites from 8 pigs, wild-type n = 11 sites from 8 pigs, $p > 0.05$ repeated measures ANOVA). This suggests that cAMP stimulation may cause hyperactive ENaC in CF.

Forskolin treatment may downregulate ENaC activity in wild-type pigs (Knowles et al., 1983, 1990; Boucher et al., 1986), which may explain the difference observed in the amiloride response. We therefore compared the amiloride response of CFTR-/- preparations pre-incubated with forskolin (CFTR-/- + forskolin) to the amiloride response of wild-type swine that were never exposed to forskolin (Fig. 3-6B). The results show a significant difference between the response to amiloride by CFTR-/- and wild-type swine (Fig. 3-6 B, $p < 0.05$ repeated measure ANOVA, CFTR-/- n = 12 sites from 8 pigs, wild-type n = 18 from 7 pigs). Amiloride treatment triggered a peak response in Na^+ flux into the ASL of 15 ± 5 nmol cm^{-2} s^{-1} in CFTR-/- preparations incubated with forskolin, and 4 ± 1 nmol cm^{-2} s^{-1} in wild-type preparations (Fig. 3-6 B). This indicates that forskolin-treated CFTR-/- swine airway display ~4 times more ENaC activity than that of untreated wild-type preparation, consistent with the hypothesis that ENaC may be hyperactive in CF. The response to amiloride by forskolin-treated CFTR-/- swine showed wide dispersion, with some sites in a single lung displaying a large response to amiloride while other sites showed responses similar to that of wild-type tissues (Fig. 3-6 C). These data suggest that some sites in the distal airway of CFTR-/- swine have hyperactive ENaC while other of the sites seems to function normally. This is consistent with the observation that early CF airway disease development is not homogeneous throughout the lung, but rather, it manifests in discrete

sites of the small airway in CFTR-/- swine (Adam et al., 2013) as well as in CF infant patients (Wielpütz et al. 2016, Sly et al. 2009).

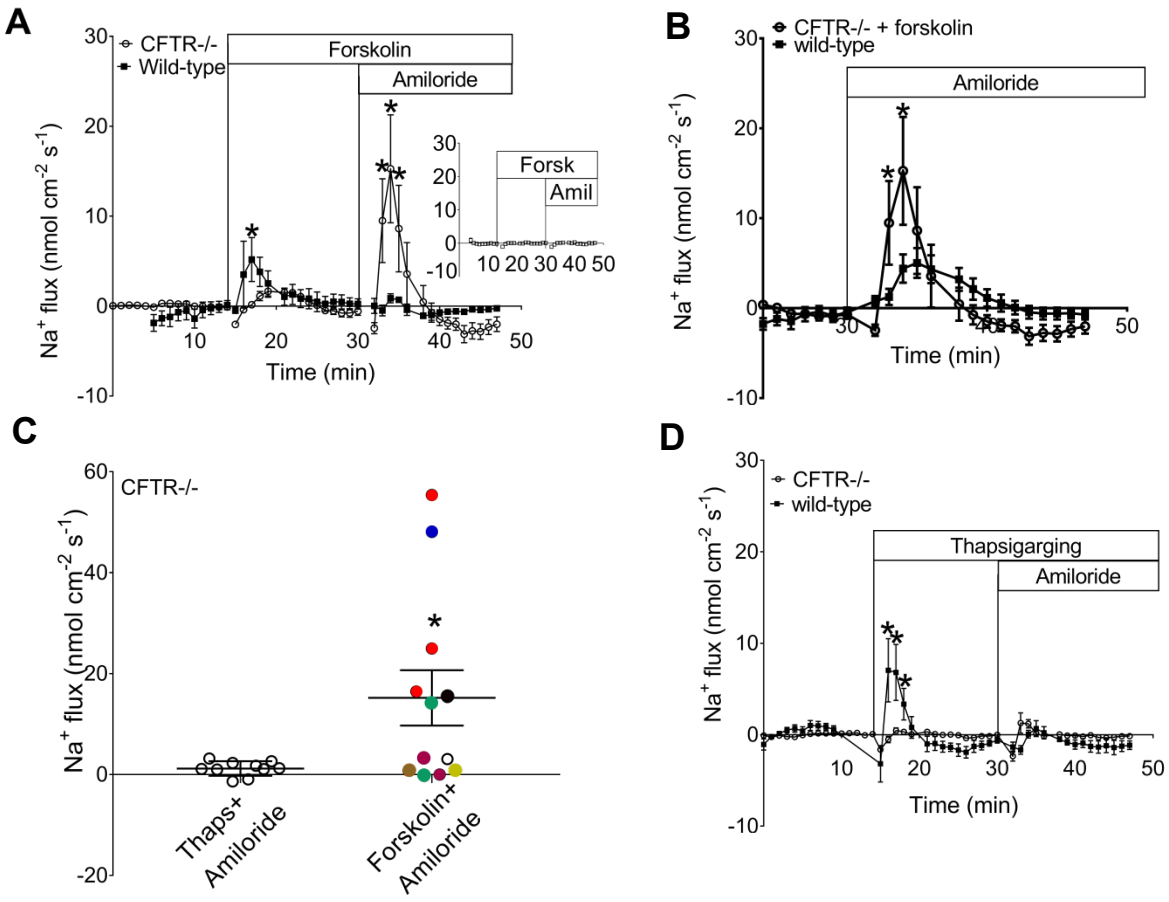

Figure 3-6: larger response to amiloride by distal airway of CFTR-/- swine. We use the response to the ENaC inhibitor, amiloride, as an assay for ENaC activity. A) Effect of forskolin on the response to amilorde by wild-type and CFTR-/- preparations. Insert display a sham experiment where the tissue was removed. B) Response to amiloride of wild-type swine that were never exposed to forskolin. C) Response to amiloride by thapsigargin-treated CFTR-/- and forskolin-treated CFTR-/- preparations. D) Thapsigargin triggers an increase in Na^+ flux into the ASL in wild-type preparations and CFTR-/- swine preparations. Asterisk denotes statistical significant difference.

To test whether ENaC hyperactivity in CFTR-/- is dependent on stimulation with Ca^{2+}, we tested the effect of amiloride on preparations treated with thapsigargin, a SERCA blocker that increases intracellular Ca^{2+} concentrations and stimulates ion flux in airway epithelia (Terran et al., 2002). Stimulating preparations with thapsigargin triggered a significantly larger increase in Na^{+} flux into the ASL in wild-type preparations than in CFTR-/- swine preparations (Fig. 3-6 D, $p < 0.05$ repeated measures ANOVA, CFTR-/- n = 10 sites from 7 pigs, wild-type n = 7 sites from 4 pigs) but amiloride produced the same response in wild-type and in CFTR-/- airways (Fig. 8 D). The response to amiloride by thapsigargin-treated CFTR-/- was significantly smaller than that of forskolin-treated CFTR-/- preparations (Fig. 3-6 C, $p < 0.05$ Students' t test with Welch's correction, forskolin+amiloride n = 12 sites from 8 pigs, thapsigargin+amiloride n = 10 sites from 7 pigs). These data suggest that ENaC hyperactivity in CFTR-/- airway is indeed dependent on cAMP stimulation.

4. Discussion

Regulation of ASL production in normal airways is a complex process, involving submucosal glands and airway surface epithelial cells. In upper airways, submucosal glands contribute up 90% (Reid, 1960) of secretion of ASL, while in small airways ASL homeostasis is carried out by airway surface epithelial cells (Butcher, 2002). Since CF airway disease starts in the distal airways, there has been great interest in studying the airway surface epithelia physiology of the distal airways (Wine 2004; Tang et al. 2016). Studying the physiology of the distal airway is difficult, due the lack of experimental techniques with sufficiently spatial resolution to measure ion transport in native tissues. Thus, most studies have relied on cell cultures of distal airway epithelia to study ion transport, which has produced very important data

(Zabner et al., 1998; Knowles et al., 2002; Li et al., 2016). However, the use of tissue cultures has limitations, including the fact that cultured cells do not necessarily have the same phenotype as native epithelia (Shamsuddin & Quinton, 2012). In addition, culture cells lack other components of the native tissue, such as neurons, and connective tissue, that may contribute to normal function. This is an important impediment to our understanding of the pathobiology of CF airway disease.

Thus, little is known about the ion transport in distal airway epithelia in native tissue. We decided to test sodium ion transport properties in CF and wild-type porcine small airways. Using SIET we investigate the Na^+ fluxes with an unprecedented spatial and time resolution. Previous studies using radio labeled ions or Ussing chambers could only produce data on the ion transport properties of the entire tissue (i.e. folds and pleats). We present the first ever direct measurement of ion transport by the folds of the airway.

The baseline activity of the small airway epithelium revealed both secretion and reabsorption of Na^+. Moreover, stimulation with forskolin produced both secretion and later reabsorption of Na^+ at a single site in the epithelium. Thus, our study revealed that the epithelium at the folds of small airway epithelia has the ability of bidirectional ion transport and rejects Quinton et al's (2012) hypothesis that folds are only capable of absorptive functions. Our data also indicates that the epithelia at the folds respond to both ENaC and CFTR pharmacological blockers, suggesting that both CFTR and ENaC are expressed in the same cells. This suggests that CFTR-ENaC interaction is, indeed, possible.

The response to amiloride by CFTR-/- pigs suggests that ENaC becomes hyperactive upon stimulation with cAMP. Thus, reconciling the enormous body of evidence of ENaC hyperactivity in CF (Hobbs et al., 2013; Althaus, 2013) with recent reports indicating normal

ENaC activity in CF tissues (Chen et al 2010, Hoegger 2014, Sun et al., 2010, Fisher et al., 2013, Tuggle at al., 2014, Itani et al., 2011). Our data also suggest that the contribution of ENaC to early disease should be tested at the sites of disease in early CF (Wielpütz, et al., 2014) and not in healthy upper airways because not all airways display the same phenotype.

4.1 Baseline Na^+ fluxes.

Baseline sodium transport (i.e. without any stimulation) by the folds of the tissue was both positive and negative Na^+ fluxes in different samples of distal airway tissues. The level of the secretion and reabsorption varied from sample to sample within the range of -100 to 100 pmol cm^{-2} s^{-1} for CF tissues and 2 to 3-fold larger for wild-type tissues. That fact that we observed opposite direction of ion fluxes over the same group of cells on the folds, strongly suggests that these cells are capable of bidirectional sodium transport. In addition, our data also shows that CF tissue displayed significantly diminished reabsorption and secretion of ions compared with wild-type, consistent with previous reports that CF tissues have lower rate of fluid transport in either direction (Birket et al., 2014).

4.2 Forskolin and thapsigargin stimulated secretion.

Forskolin stimulates the production of c-AMP that phosphorylates the regulatory domains of CFTR and opens the channel. In CF, the function of CFTR is lost; thus, we would expect CF airways to be unresponsive to treatment with forskolin, and, thus not trigger secretion of ASL. Surprisingly, we recorded forskolin dependant increase of Na^+ fluxes in CF, which in to cases were close to the mean of forskolin response in wild-type. The fact that forskolin could induce outward Na^+ transport in CF airways might be the result of cAMP mediated activation of

basolateral K^+ channels (Lohrmann et al., 1995; MacVinish, 1998), thus hyperpolarizing the membrane potential of the apical membrane, thus driving Na^+ in to the lumen. Alternatively, cAMP may result in increased intracellular Ca^{2+} level and activation of Ca^{2+}-activated Cl^- channels (CACC) and increase ion secretion. However, the average of CF forskolin induced secretion was significantly lower than in WT.

We tried to activate the CACC-dependent Cl^- pathway by stimulating intracellular Ca^{2+} increase with thapsigargin, a SERCA blocker, to increase intracellular calcium. The data showed that stimulation with thapsigargin failed to invoke any significant changes in sodium transport in CF epithelia. However, WT samples stimulated with thapsigargin consistently showed elevation in Na^+ fluxes.

4.3 Amiloride inhibition of ENaC

According to the previous studies (Zhou et al., 2011, Gaillard et al., 2010) CF epithelium might express hyperactive epithelial sodium channels (ENaC). We, therefore, tested the response of CFTR -/- to the ENaC inhibitor, amiloride, as an assay for Na^+ transport since inhibiting ENaC in an epithelium that is capable of bidirectional NaCl transport would reveal the secretion component (Phillips et al., 1999). Moreover, the size of the amiloride response would directly depend on the ENaC activity before amiloride treatment. This proxy measurement (i.e. response to amiloride) has been used before to study ENaC function in the upper airway of CFTR-/- animal models (Chen et al., 2010, Sun et al., 2010, Fisher et al., 2013, Tuggle at al., 2014) and in CF human epithelial cell culture (Boucher et al., 1986, Itani et al., 2011). Pre-incubating wild-type preparations with forskolin and then treating them with amiloride produced an increase in Na^+ secretion into the PCL, which is consistent with previous reports showing that blocking ENaC increases secretion of fluid into the PCL (Blouquit et al. 2006). The response to amiloride

is as expected from an epithelium that is capable of bidirectional transport and the net fluid transport results from a balance between secretion, which is mediated by Cl^- channels, and reabsorption, which is mediated by ENaC (Phillips et al., 1999).

We observed that some localized regions showed amiloride induced higher than normal secretion in CFTR-/- tissue. Based on this result, we conclude that some regions of CF small airways might contain hyperactive ENaC, while others may not. This result is consisted with previous research suggesting increased reabsorption of Na^+ in CF (Boucher et al. 1986). Boucher and colleagues also reported that in CF respiratory epithelia, hyperabsorption is not homogenously observed, but rather some localized spots in airway epithelia might express hyperactive ENaC.

5. Conclusion

Adequate hydration of the PCL is essential for efficient mucociliary clearance, the very basis of the lung's innate defence system. A large body of evidence suggest that PCL volume is regulated to an optimal level by airway surface epithelial cells that are capable of bidirectional fluid transport (Phillips et al., 1999, Chamber et al., 2007), i.e. CFTR-mediated PCL secretion and ENaC-mediated PCL reabsorption. In normal airways CFTR inhibits ENaC, thus downregulating PCL reabsorption. In CF this process fails and PCL becomes dehydrated. This model has been recently challenged by reports that CFTR-/- do not display ENaC hyperactivity, and that the airway epithelial cells are not capable of bidirectional transport.

Our data seems to show that airway surface epithelia are capable of bidirectional transport. In addition, it shows that CFTR-/- airway display higher than normal amiloride response in some sites ENaC after forskolin stimulation, suggesting hyperactive ENaC.

Based on our data and published reports the pathobiology of CF airway disease seems to by multi causal. There are ASL volume and properties dysfunction in CF, which favors the persistence of pathogens. In the distal airway, signals that would normally stimulate ASL secretion (such as cAMP) instead trigger hyperactive ENaC. One such signal may be the presence of pathogens. Hyperactive ENaC would cause mucociliary clearance collapse, failure of the innate immune system, and infection. Repeated cycles of infection and inflammation would trigger tissue remodeling that may eventually lead to respiratory failure.

6. Appendix I: Tissue viability

Concerns about tissue viability were addressed. We investigated the baseline sodium fluxes obtained during at different times after euthanasia. There was no correlation between the fluxes and the time after euthanasia up to 18 h. Since we performed our experiments within 12h after euthanasia, the tissue was viable and healthy.

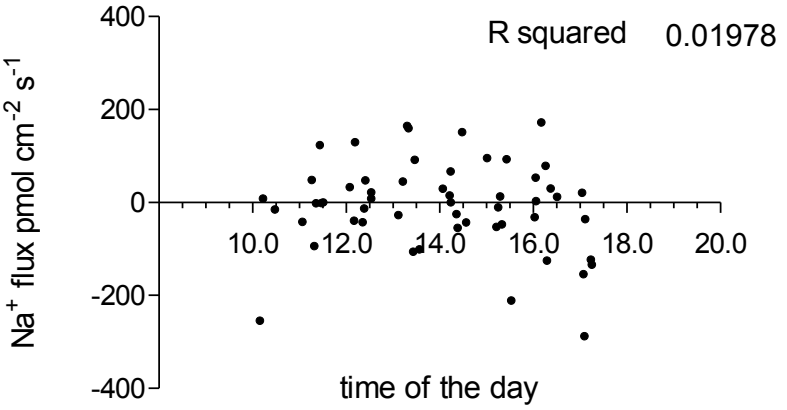

Figure 6-1: Correlation of baseline sodium flux and time after euthanasia in WT swine.

Data points are averages of baseline Na^+ fluxes recorded within 10 min in different samples at different times after euthanasia (N=54 p=0.3104, R squared=0.01978).

7. Appendix II: Complete data on the effects of thapsigargin

Ion and fluid transport by the airway surface epithelia can also be modulated by the intracellular second messenger, Ca^{2+} (Liedrke et al., 1989). Increases in intracellular Ca^{2+} trigger increases in Na^{+} and Cl^{-} transport into the ASL through Ca^{2+} activated Cl^{-} channels (CaCC) (Schreiber et al., 2005; Ruppersburg,& Hartzell. 2014) that can be stimulated with the SERCA blocker, thapsigargin (Ishii et al 1994; Lytton et al. 1991). We tested the role of Ca^{2+}-dependent pathways on ion flux by stimulating our preparation with thapsigargin (1 μM, Terran et al., 2002). Wild-type swine airway preparations responded to thapsigargin with a transient increase in Na^{+} flux into the ASL that peaked at 5 ± 2 min after treatment at 508 ± 102 pmol cm^{-2} s^{-1}, which was significantly higher than the baseline secretion, then the tissues reversed to Na^{+} reabsorption from the ASL (Fig. 7-1 A). Preparations incubated with the CFTR inhibitor, CFTRinh172 (100 μM), responded no differently to thapsigargin than untreated preparations, suggesting a minimal contribution of CFTR to thapsigargin-stimulated Na^{+} flux (Fig. 7-1 C and D). However, CFTR-/- swine epithelia had a significantly smaller response to thapsigargin than that displayed by wild-type tissues and tissues incubated with the CFTR inhibitor (Fig. 7-1 B). This is an unexpected result since there is evidence that CFTR and CaCC functionally interact in such a way that CFTR reduces CaCC currents (Wei et al., 1999) and accordingly, CaCC is up regulated in CFTR-/- mouse tracheal epithelial cell cultures (Tarran et al. 2001). We therefore expected that thapsigargin response in CFTR-/- swine preparations would be larger than that in wild-type animals. Our results to the contrary would suggest that CaCC may be downregulated in CFTR-\- swine. CF airways may downregulate CaCC in response to increased levels of TGF-beta in CF airways (Salvatore 2002). This may explain why human CF airway cells have a smaller response to the CaCC blocker, DIDS, compared to normal cells (Itani 2011) and also the

reduced response to cholinergic stimulation by submucosal glands in CFTR-/- swine airways (Wine, & Joo, (2004). The reduced CaCC function in CF airways may contribute to reduced ASL secretion in CF airways.

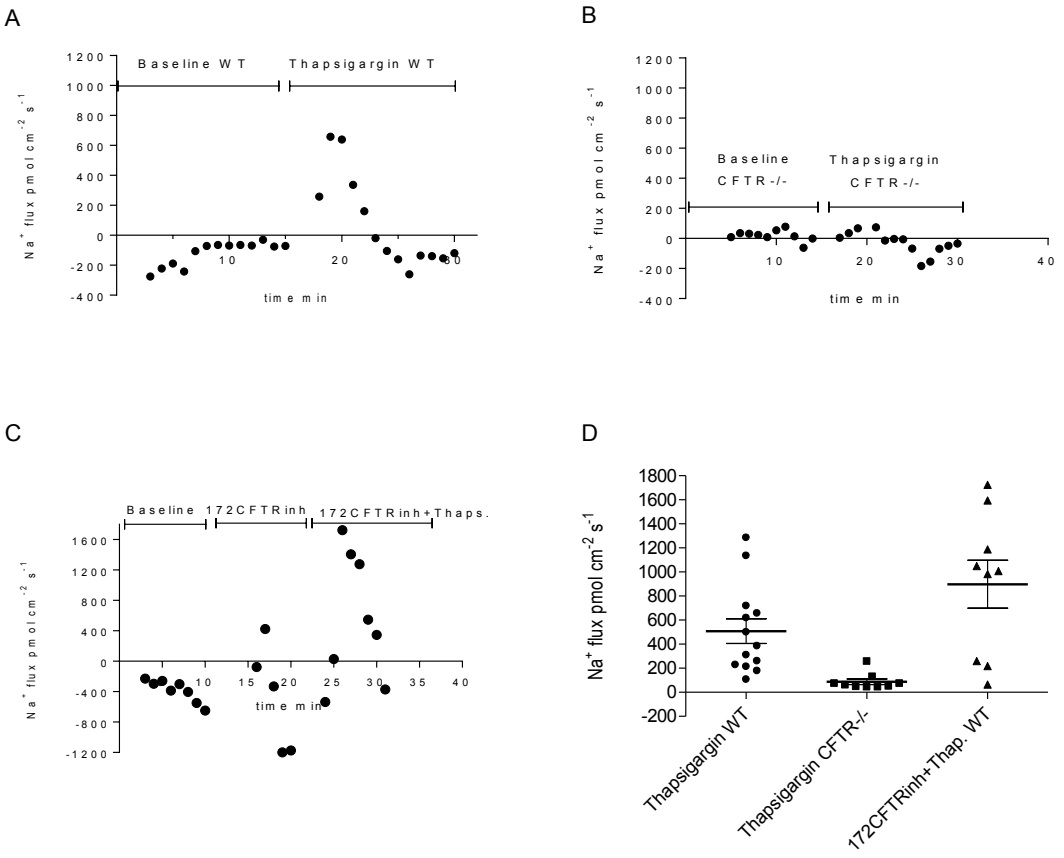

Figure 7-1: **Effects of thapsigargin in CF and WT tissue.** A. Thapsigargin stimulation of Na^+ fluxes in wild-type airway epithelia. B. Effect of thapsigargin on CFTR-/- epithelia. C. Effect of thapsigargin CFTRinh172-treated wilt-type epithelia. D. Statistical analysis of thapsigargin responses in CF and wild-type epithelia showing significantly smaller response by CF epithelia (n =13,9,9; $p<0.0004$; non-parametric Kruskal-Wallis test)

8. Appendix III: There is no difference in ion transport between 1week-old and 2-3 week-old animals

Since some data was obtained from 2-3 weeks-old swine. Our concern was if this data is consistent and comparable to results recorded from 2-7 day-old CFTR-/- samples. Thus, we compared the two age groups using the same drugs utilized in CFTR-/- experiments. Our data revealed that 1 week-old and 2-3 weeks-old animals have the same response to forskolin, thapsigargin, and amiloride (Fig. 8-1). Thus there is no effect of age of the animals on our results.

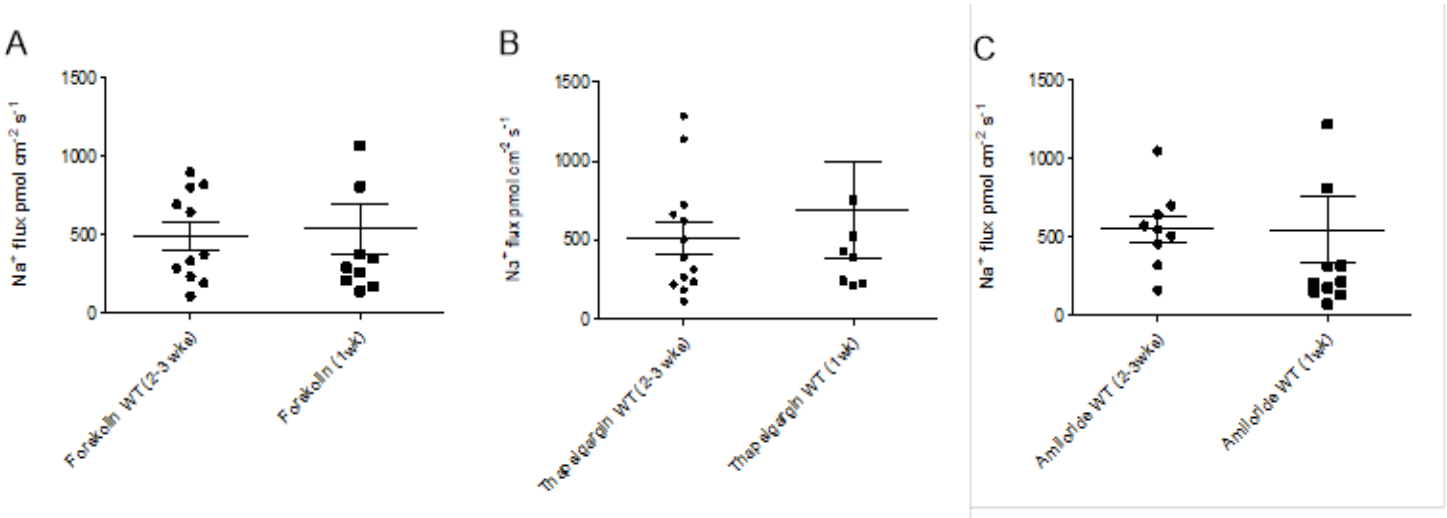

Figure 8-1: Comparisons of drugs responses in tissue from 1 week-old (1wk) and 2-3 weeks-old (2-3 wks) pigs. A. Forskolin induced stimulation of Na^+ secretion in 1wk and 2-3 wks old WT pigs (n=10;11, p=0.9159, Mann Whitney test). B. Na^+ fluxes analysis in two groups WT tissue 1 and 2-3 wks old pigs (n=8; 13, p= 0.9135, Mann Whitney test). C. Amiloride-conditioned Na^+ secretion in porcine distal airways from two different age groups (n=11: 9, p= 0.1715).

9. Appendix IV: Mouse small airway

While developing the pig model, I have simultaneously attempted to test the same hypothesis using mice small airways. Using mice as a model has several advantages. Firstly, it is more convenient to obtain mice and euthanize them immediately before using. Another advantage is the availability of many genetically modified mice to test different aspects of ASL secretion including CFTR- or ENaC- knockout mice.

Although the mouse model seems to have lot of beneficial aspects, it turns out that it is difficult to perform these experiments in mice. The problem is that the mice's small airways (1-2mm in diameter) don't have the same clear and distinct anatomical architecture of the epithelial layer as the swine model does. Thus, it is not possible to determine the pleats from the folds. Despite this disadvantage I continued using mice airways in order try to identify differences in ion flux properties (Fig. 9-1). Producing a frequency distribution, I did not find two populations of data as one would expect if there were a group of cells secreting and another absorbing Na^+. In contrast the data distributed around 0 flux. Addition amiloride or NaN3, to inhibit active transport, resulted in a distribution tighter around 0.

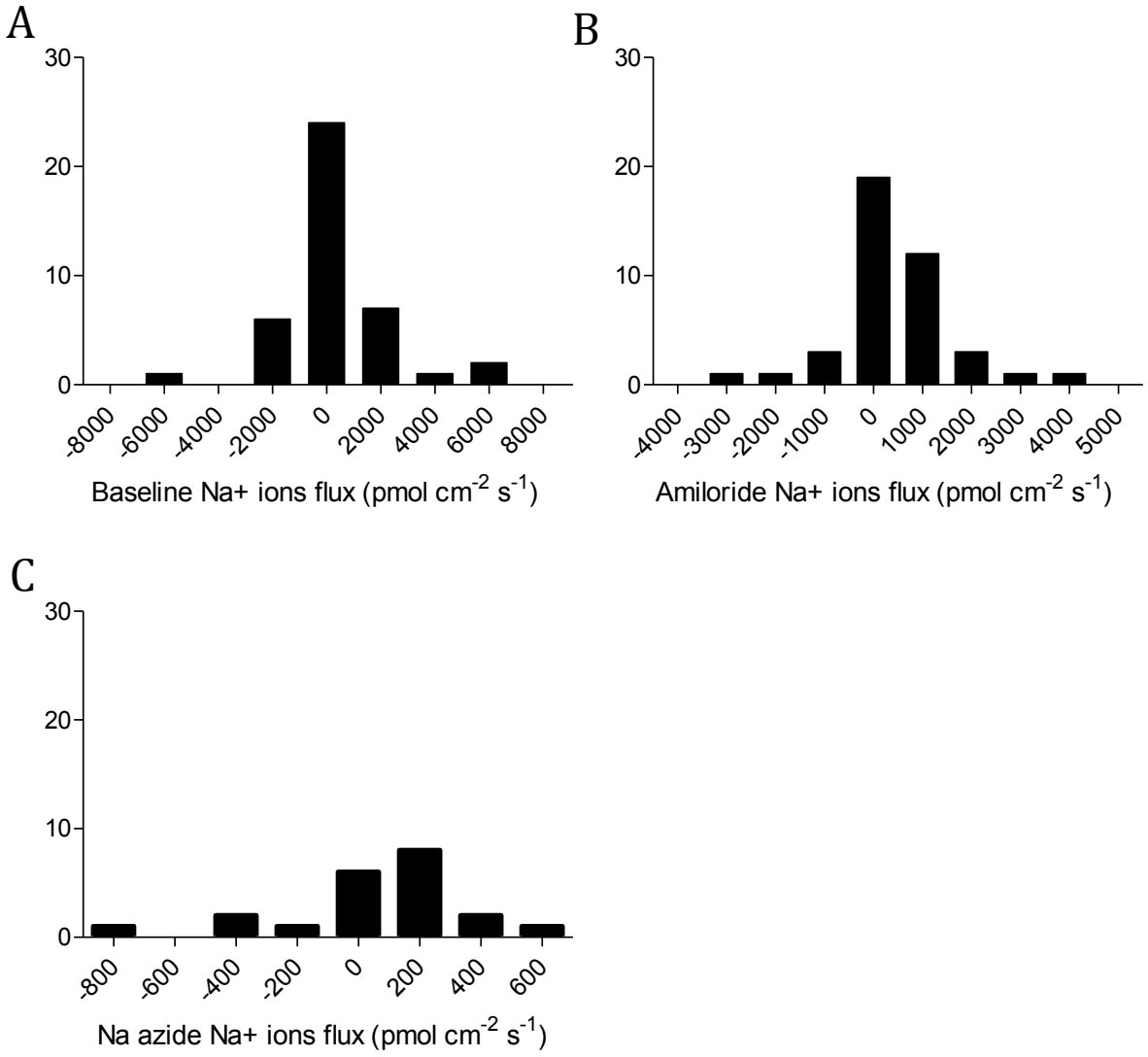

Figure 9-1: Frequency distributions of Na^+ flux in mice. A. Baseline distribution obtained in mourine distal airway epithelia (n=41). Data showed vague displacement of recordings with 90% of the data points were laid between -2000 and 2000 pmol cm^{-2} s^{-1}. B. Amiloride effects on distribution of the Na+ fluxes in mouse small airways (n=41). C. Na azide modulations of the Baseline secretions of sodium, narrowed data points displacement within -800 to 600 pmol cm^{-2} s^{-1} range (n=21).

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