THE ROLE OF K^+ CHANNELS IN CELL PROLIFERATION AND CELL VOLUME REGULATION IN HUMAN BRONCHIAL EPITHELIAL CELLS

by

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ABSTRACT

K^+ channels are expressed in a wide variety of cell types, including airway epithelial cells. The purpose of this study was to investigate the role of various K_v and K_{Ca} channels in cell proliferation and cell volume regulation in HBE and CFBE cell lines. Methods included mRNA and protein detection, cell counting and cytotoxicity assays, and cell volume experiments. HBE and CFBE cells expressed many K^+ channel mRNA and protein, including K_v7.1, K_v7.5, K_v4.1, K_v4.2, K_v10.1, K_v11.1, K_{Ca}1.1 and K_{Ca}3.1. Various K_v and K_{Ca} channels played a role in HBE and CFBE basal cell proliferation, as well as in mediating the regulatory volume decrease (RVD) response in HBE cells. CFBE cells did not elicit an RVD response and neither cell line underwent a regulatory volume increase (RVI) response. Therefore, K^+ channels play an important role in cell proliferation and the regulation of cell volume in bronchial epithelial physiology.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ASL	airway surface liquid
α	alpha
NH_2	amino
bp	base pair
β	beta
HCO_3^-	bicarbonate
K_{Ca}	calcium-activated potassium channel
CaM	calmodulin
COOH	carboxyl
°C	celsius
cm	centimeters
CNS	central nervous system
CFBE	CFBE41o-
Cl^-	chloride
cDNA	complementary deoxyribonucleic acid
[C]	concentration
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator gene
dNTP	deoxyribonucleoside triphosphate
DMSO	dimethyl sulfoxide
S	DNA synthesis
elk	eag-like
erg	eag-related
EGFP	enhanced green fluorescent protein
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ENaC	epithelial Na^+ channel
E2	17β-estradiol
eag	ether-a-go-go
EBIO	1-ethyl-2-benzimidazolinone
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
G_1	first gap
GFG	glycine-phenylalanine-glycine
GYG	glycine-tyrosine-glycine
g	gram
IC_{50}	half maximal inhibitory concentration
HBE	16HBE14o-
hr	hours
hyper	hyperosmotic
hypo	hyposmotic
IGF-1	insulin-like growth factor-1

IGF-1R	insulin-like growth factor-1 receptor
$[Ca^{2+}]_i$	intracellular calcium concentration
$[K^+]_i$	intracellular potassium concentration
$[Na^+]_i$	intracellular sodium concentration
K_{ir}	inward-rectified potassium channel
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium
iso	isosmotic
kDa	kilodaltons
LDH	lactate dehydrogenase
L	litres
mRNA	messenger ribonucleic acid
m	meters
μ	micro, 10^{-6}
m	mili, 10^{-3}
MEM	Minimum Essential Media
min	minutes
M	mitosis
M	molar
mol	mole
M-MLV	Moloney Murine Leukemia Virus
MCC	mucociliary clearance
M-type	muscarine sensitive
I_m	muscarine sensitive potassium current
n	nano, 10^{-9}
Opti-MEM	Opti-MEM Reduced Serum Media
%	percent
PCL	perciliary liquid layer
PMSF	phenylmethanesulfonyl fluoride
4α-PDD	4α-phorbol 12, 13-didecanoate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
K_2P	2-pore potassium channel
K^+	potassium
KCC	potassium/chloride co-transporter
1°	primary
I_{Kr}	rapid delayed rectifier potassium current
RVD	regulatory volume decrease
RVI	regulatory volume increase
RCF	relative centrifugal force
RT	reverse transcription
RPM	revolutions per minute
2°	secondary
s	seconds
G_2	second gap
I_{Ks}	slow delayed rectifier potassium current
siRNA	small interfering ribonucleic acid

Na^+	sodium
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
t	time
I_A	transient outward A-type potassium current
I_{to}	transient outward potassium current
TMDs	transmembrane domains
TBS	Tris-base saline
TBS-T	Tris-base saline with Tween
TBE	Tris/Borate/EDTA
U	units
$^v/_v$	volume to volume
K_v	voltage-dependent potassium channel
V	volts
H_2O	water

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CHAPTER 1: INTRODUCTION

1.1 Epithelial cell physiology

Epithelial cells line various organs in the human body and are involved in many physiological functions, including barrier formation and the regulation of body fluid composition and volume through ion transport (Wills *et al*., 1996). These mechanisms require epithelial cells to have two very important properties, which are characteristic to all types of epithelial cells; firstly, the fact that they have tight junctions, and secondly, their ability to be polarized, which means they have transport proteins asymmetrically distributed between their apical and basolateral membranes (Wills *et al*., 1996). The tight junctions are essential for the barrier role that epithelial cells play, because they allow the cell to control the passage of not only fluids, but also ions and other solutes between adjoining cells, creating a route of transport between two neighbouring cells known as the paracellular pathway (Wills *et al*., 1996). Due to the polarization of epithelial cells, they are able to transport ions and fluids in order to regulate the composition and volume of body fluids (Muth and Caplan, 2003). These characteristics allow for the transepithelial transport of ions and fluids, one of the most studied aspects of epithelial cell physiology. (Muth and Caplan, 2003).

In the airways, and more specifically the bronchi, epithelial cells not only play a role in barrier formation and ion transport, but also in defense mechanisms such as mucociliary clearance (MCC) and innate host defense, as well as in physiological processes like wound repair, cell proliferation and cell volume regulation (Wills *et al*., 1996; Chilvers and O'Callaghan, 2000; Trinh *et al*., 2007). Since the respiratory system is continuously exposed to the external environment, airway epithelial cells develop defense mechanisms in order to protect the lungs from inhaled pathogens and infection. The main line of defense in the bronchi is MCC, where apically located cilia that line the epithelium physically get rid of any unwanted material (Chilvers and O'Callaghan, 2000). The bronchial epithelial cells are covered with airway surface liquid (ASL), that contains a heavier top mucus layer that traps pathogens that have been inhaled into the airways, and a more liquid lower layer known as the periciliary liquid layer (PCL), that bathes the cilia and allows them to beat properly and in an orderly fashion. During MCC,

the cilia beat in a coordinated manner and move the mucus layer of the ASL to the pharynx so it can either be swallowed or coughed out (Sleigh *et al*., 1988). Bronchial epithelial cells also play a role in innate host defense, since they have the ability to release immunoglobins and other antimicrobial agents into the secretions that line the airways, and thus protect the lungs against infection (Gerritsen, 2000). In order for MCC to function properly and keep the lungs healthy and clean, the PCL volume must be closely regulated by ion and fluid transport (Blouquit-Laye and Chinet, 2007). Therefore, as mentioned above, one of the main functions of epithelial cells in the airways, and probably the most studied, is their ability to control the ionic composition and volume of fluids at the surface of the airways, also known as the transepithelial transport of ions and liquids. A classical example of a dysfunction in this transport mechanism is seen in the disease cystic fibrosis (CF).

1.2 Ion channels in the airways and cystic fibrosis

The level of ASL in normal airway epithelial cells allowing for MCC is maintained by a balance between sodium (Na^+) absorption and chloride (Cl^-) secretion. This balance creates an adequate PCL volume, which allows for proper transport of ions and fluid, and keeps the lungs healthy via effective MCC. In the airways of an individual suffering from CF, the balance between Cl^-secretion and Na^+ absorption is offset and causes a reduction of the PCL volume and an increase in sticky, dehydrated mucus (Bardou *et al*., 2009). CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator gene (CFTR), which encodes an apically located Cl^- channel that shares the same name (Kerem *et al*., 1989). To date, there are over 1,800 different CF-causing CFTR mutations; however the most common is the ΔF508 mutation (Cystic Fibrosis Canada, 2011). This mutation is a three base pair (bp) deletion that omits a phenylalanine amino acid from position 508 on chromosome 7 and causes the CFTR protein to improperly fold and degrade before it is transported to the cell membrane (Ward *et al*., 1995). Because CFTR accounts for the majority of anion secretion in airway epithelial cells, mutations in this gene decrease these secretions, including Cl^- and bicarbonate (HCO_3^-), and increase the absorption of Na^+ ions through the apical epithelial Na^+ channel (ENaC), in turn disrupting the ionic balance (Gadsby *et al*., 2006).

The CFTR protein is expressed on the apical surface of many epithelial cells, including those in the nose, colon, and lungs (Riordan *et al*., 1989). In the lungs, most of the CFTR is expressed in the serous cells of the submucosal glands; however, there is expression of this protein in the surface epithelium that lines the airways (Jiang and Engelhardt, 1998). In people who suffer from CF, the decrease in Cl^- secretion and alternately the hyperabsorption of Na^+, leads to dehydrated mucus due to the lack of water following the transport of salt (see below and Figure 1). This accumulation of dehydrated and sticky mucus together with decreased PCL volume means that beating cilia are not as effective at removing mucus from the lungs, a breeding ground for pathogens is established and potentially lethal infections can develop (Gadsby *et al*., 2006).

1.3 K^+ channels in epithelial cell physiology

The most studied airway epithelial ion channels are currently CFTR and ENaC, due to the fact that Cl^- and Na^+ are the dominant transepithelial players in respiratory cell physiology, and the major candidates under investigation for CF therapies (Bardou *et al*, 2009). Potassium (K^+) channels have been of less focus, even though about 40 types have been identified in airway and alveolar epithelial cells (Bardou *et al*; 2009). One of the most important roles that these channels play in epithelial cells is their ability to control membrane potential, and in turn, maintain the electrochemical gradient for the transport of ions and fluids (Salkoff *et al*., 2006). The presence of Na^+/K^+-ATPase pumps cause a greater intracellular K^+ concentration ($[K^+]_i$), leading to an overall efflux of K^+ from the cell when selective K^+ channels are open, which in turn hyperpolarizes the cell membrane (Muth and Caplan, 2003). K^+ channels are also important for the absorptive properties of epithelial cells, and those lining the bronchi are involved in Na^+ absorption from the lumen into the cell (Muth and Caplan, 2003). As Na^+ is pumped out of the cell via Na^+/K^+-ATPase pumps on the basolateral membrane, K^+ is pumped into the cell. The intracellular K^+ then leaves the cell through apically and basolaterally located K^+ channels (Bernard *et al*., 2003; Moser *et al*., 2008). Na^+ is then able to move freely into the cell when Na^+ channels open on the apical membrane, due to the increased gradient for Na^+ that the Na^+/K^+-ATPase pump has created (Figure 1).

K^+ channels in epithelial cells are also responsible for creating the driving force for anion secretion through their ability to hyperpolarize the cell membrane (Smith and Frizzell, 1984; Cuthbert *et al*., 2003). $Na^+/K^+/2Cl^-$ co-transporters on the basolateral membrane transport Na^+, K^+ and Cl^- ions into the cell. Na^+ is removed via Na^+/K^+-ATPase pumps also located on the basolateral membrane, which add more intracellular K^+. When K^+ channels on the apical and basolateral surface are activated, K^+ exits the cell, moving down its concentration gradient. This causes the cell to hyperpolarize, driving anions such as Cl^- and HCO_3^- out through CFTR channels on the apical membrane (Figure 1) (Devor *et al*., 1999). This close relationship between K^+, Cl^-, and Na^+ allows for the possibility of pharmacological treatments that target K^+ channels in diseases such as CF.

K^+ channels are classified into 3 different groups according to the number of transmembrane domains (TMDs) the channel possesses (Bardou *et al*., 2009). The first group encompasses K^+ channels with 2 TMDs and includes the inward-rectifier K^+ channels (K_{ir}), such as $K_{ir}6.1$. The second group consists of K^+ channels with 4 TMDs and contains the 2-pore K^+ channels (K_2P), which includes the members Twik -1, -2, Trek -1, -2, and Task -2, -3, -4. The final group, and of interest to this study, involves K^+ channels with 6 TMDs, which consists of two subgroups, the voltage-dependent (K_v) and calcium (Ca^{2+})-activated (K_{ca}) K^+ channels. The K^+ channel names and the genes they encode that are of interest to this project are described in Table 1.

1.4 Voltage-gated (K_v) K^+ channels

One of the largest families of K^+ channels are the K_v channels, which are known to include some of the most studied K^+ channels in the airway epithelium (Bardou *et al*., 2009). These channels possess a tetramer of 6 TMDs and a single pore loop between S5 and S6 that contains the highly conserved glycine-tyrosine-glycine (GYG) amino acid signature that forms the K^+ selectivity filter, except for in $K_v11.1$, which has a glycine-phenylalanine-glycine (GFG) signature (Heginbotham *et al*., 1992; Pongs, 1992; Bardou *et al*., 2009). They also have intracellular amino (NH_2)- and carboxy (COOH)- terminals, and their S4 TMD is an important player in sensing voltage changes in the cell membrane (Figure 2A) (Benzanilla, 2000; Robbins, 2001). Although $K_v7.1$ has gained much

attention as playing an important role in cardiac physiology since it is the main player in the disease Long QT1 syndrome (Sanguinetti and Jurkiewicz, 1990), K_v7.5, members of the K_v4 family, K_v10.1, and K_v11.1 have also surfaced as being interesting channels in epithelial cell physiology (as described below), and require further exploration.

1.4.1 K_v7.1 and 7.5

The K_v7 family includes five members, K_v7.1-7.5, which have been reported to associate with minK proteins, also known as KCNE subunits, and are specific beta (β) accessory subunits that can modify the channel's biophysical characteristics (Sanguinetti *et al*., 1996; Robbins, 2001). For example, in the heart K_v7.1 forms a complex with KCNE1 and generates the slow delayed rectifier K^+ current (I_{Ks}) (Sanguinetti and Jurkiewicz, 1990). K_v7.1 has been intensely studied in cardiac physiology, where it was first identified in a linkage study that was investigating cardiac arrhythmias, and mutations in this channel can lead to the heart arrhythmia Long QT1 syndrome (Sanguinetti and Jurkiewicz, 1990; Wang *et al*., 1996). Multiple pharmacological inhibitors exist for these channels including linopirdine and its analogue XE991, which inhibit all five K_v7 members, and chromanol 293B, which selectively inhibits K_v7.1 (Warth *et al*., 1996; Wang *et al*., 1998; Zaczek *et al*., 1998). It is more difficult to find suitable activators for these channels, however the Cl^- channel blocker DIDS and mefenamic acid have been proposed as potential K_v7 channel activators (Busch *et al*., 1994; 1997).

K_v7.1 expression has been detected in bronchial epithelial cells, where it is activated by intracellular cyclic adenosine monophosphate (cAMP) (Mall *et al*., 2000). In Calu-3 cells, which are a widely used model of airway submucosal gland serous cells, it has been concluded that K_v7.1 channels on the apical and basolateral membranes play an important role in the cell's basal and cAMP-activated anion secretion (Cowley and Linsdell, 2002; Moser *et al*., 2008).

K_v7.5, another member of the K_v7 channel family, has been less studied in airway epithelial cells. In the brain it plays a role in selectively transporting K^+ ions and contributing to the muscarine sensitive (M-type) K^+ current (I_m) (Robbins, 2001). Although K_v7.2 and K_v7.3 have been suggested as the K_v7 members responsible for the

slow activation and lack of inactivation characteristics of this current, $K_v7.4$ and $K_v7.5$ are also expressed in the brain and show similar M-current characteristics (Kubisch *et al*., 1999; Lerche *et al*., 2000). Because $K_v7.5$ can co-assemble with $K_v7.3$, it has been suggested that $K_v7.5$ also contributes to the I_m current (Lerche *et al*., 2000). $K_v7.5$ has also been reported to play a role in cell proliferation in skeletal muscle (Roura-Ferrer *et al*., 2008), and along with $K_v7.3$ it reportedly plays an important role in Na^+ transport in airway epithelial cells (Greenwood *et al*., 2009).

1.4.2 K_v4.1, 4.2 and 4.3

Like the K_v7 family of K^+ channels, the majority of research on members of the K_v4 family has been performed in the nervous and cardiovascular systems (Birnbaum *et al*., 2004). They have the same topology as described for K_v7 channels (Figure 2A). In mammals K_v4 channels have three family members; $K_v4.1$, $K_v4.2$, and $K_v4.3$ (Birnbaum *et al*., 2004). These channels have been reported to interact with various auxiliary subunits and scaffolding proteins including KChIPs on the NH_2-terminal end of the channel, and $K_v\beta$ subunits on the COOH-terminal end (Barry *et al*., 1998; Bahring *et al*., 2001). In the nervous system it has been reported that these channels contribute to the transient outward A-type K^+ current (I_A), whereas in the cardiovascular system they contribute to the transient outward (I_{to}) current (Birnbaum *et al*., 2004). Inhibitors of these channels include heteropodatoxins and phrixotoxins (Sanguinetti *et al*., 1997; Diochot *et al*., 1999).

In the lungs all three members of the K_v4 channel family express messenger ribonucleic acid (mRNA) in the alveolar epithelium, whereas only $K_v4.2$ and 4.3 protein have been detected (Lee *et al*., 2003). $K_v4.2$ and 4.3 have also been reported as being apically expressed, and $K_v\beta1.1$, 2.1, and 3.1, as well as KChIP2 and 3 are also expressed in these tissues (Lee *et al*., 2003). There is little known about the function of these channels in the respiratory epithelium; however, it has been suggested that they may be involved in K^+ recycling (Lee *et al*., 2003), oxygen sensing (O'Grady and Lee, 2003), and volume regulation (Harron *et al*., 2009). Knowing their roles in bronchial epithelial cells may give insight towards their place in respiratory physiology.

1.4.3 K_v10.1 and 11.1

K_v10.1 is a member of the EAG family of K_v channels, which follows the typical K_v channel topology (Figure 2A). There are three subfamilies within EAG; eag (ether-a-go-go), elk (eag-like), and erg (eag-related), and the eag sub-family includes eag1, or K_v10.1 (Bauer and Schwarz, 2000). These channels have been found to co-localize with the accessory subunits minK and miRP1 (McDonald *et al*., 1997; Abbott *et al*., 1999), and contribute to a slowly activating, non-inactivating outward current (Bauer *et al*., 2001). Inhibitors of this channel include the anti-histamine astemizole and the anti-depressant imipramine (Garcia-Ferreiro *et al*., 2004). K_v10.1 has been reported to play a role in myoblast differentiation and the cell cycle (Occhiodoro *et al*., 1998), while dysfunctional K_v10.1 can cause increased neural excitability (Ganetzky and Wu, 1983). This channel has received attention as potentially playing an important role in cancer because it is aberrantly expressed in many cancerous tissues including the skin, breast, and colon (Meyer *et al*., 1999; Pardo et al., 1999; Ousingsawat *et al*., 2007). In MCF-7 cells, a widely used cell line of cancerous mammary epithelial cells, K_v10.1 plays a role in the progression of the cell through the G_1 phase of the cell cycle and cell proliferation (Ouadid-Ahidouch *et al*., 2001; Roy *et al*., 2008).

K_v11.1 is a member of the erg sub-family of EAG K_v channels, and is most noted as contributing to the rapid component of the cardiac delayed rectifier K^+ current (I_{Kr}), as well as playing a major role in the repolarization phase of the cardiac action potential (Sanguinetti *et al*., 1995). Inhibitors of this channel include the anti-arrhythmic drug E-4031 (Yao *et al*., 2005) and the scorpion toxin Ergtoxin (Scaloni *et al*., 2000). The effects that this channel has in cardiovascular physiology have led to the discovery that a loss-of-function mutation in K_v11.1 causes Long QT syndrome 2 (Curran *et al*., 1995). K_v11.1 has been reported to play a role in pacemaker activity in interstitial cells of Cajal (Zhu *et al*., 2003), along with insulin secretion from pancreatic β-cells (Rosati *et al*., 2000). It has also been detected as being aberrantly expressed in multiple primary carcinomas but not in their normal counterparts (Bianchi *et al*., 1998; Cherubini *et al*., 2000; Smith *et al*., 2002; Wang *et al*., 2002a). In MCF-7 cells, K_v11.1 channels play a role in cell volume regulation (Roy *et al*., 2008), making them interesting players in epithelial physiology.

1.5 Calcium-activated (K_{Ca}) K^+ channels

K_{Ca} channels are expressed in many excitable and non-excitable tissues, and are gated by either both membrane potential and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), or solely $[Ca^{2+}]_i$ (Vergara *et al*., 1998). The K_{Ca} channel family contains two subfamilies: Slo and SK channels, which differ in their conductance and gating properties. $K_{Ca}1.1$ is a member of the Slo subfamily (Slo1), and has a large unitary conductance. It is also gated by both $[Ca^{2+}]_i$ and membrane depolarization (Sah, 1996). On the other hand, $K_{Ca}3.1$ is a member of the SK subfamily (SK4), has a smaller unitary conductance and is gated by $[Ca^{2+}]_i$ only (Sah, 1996).

1.5.1 $K_{Ca}1.1$

The $K_{Ca}1.1$ channel has similar topology to K_v channels; however it also differs in various ways. It is a tetrameric structure but it contains an additional TMD (S0) (Berkefeld *et al*., 2010). This causes the NH_2-terminal end to relocate to the extracellular side of the channel while the COOH-terminal end remains on the intracellular side (Figure 2B). This COOH-terminal end is much larger than that seen in typical 6TMD K^+ channels and is the location of the Ca^{2+} binding site that allows for channel activation (Meera *et al*., 1997; Magleby, 2003). Since $K_{Ca}1.1$ is also activated by membrane depolarization, its voltage sensor is still under investigation, however it has been suggested that the TMDs S2-S4 play a role in this biophysical mechanism (Pantazis *et al*., 2010). Most $K_{Ca}1.1$ channels are associated with the β-subunits BKβ, which play a role in mediating the channel's signaling (Berkefeld *et al*., 2006). This channel is activated by benzimidazolones, including NS004 and NS1619, and inhibited by the scorpion toxins iberiotoxin and charybdotoxin, as well as the mycotoxin, paxilline (McKay, 1994; Holland *et al*., 1996; Strobaek *et al*., 1996; Kraft *et al*., 2003; Wei *et al*., 2005). Physiologically these channels have been suggested to play a role in many areas, including neuronal after-hyperpolarization (Faber and Sah, 2003), oxygen sensing in the carotid bodies (Williams *et al*., 2004), as well as K^+ secretion in the kidneys (Pluznick and Sansom, 2006). They have also been reported as being involved in cell proliferation in breast cancer (Ouadid-Ahidouch *et al*., 2004), and regulating cell volume in the presence of hyposmotic shock in bronchial epithelial cells (Fernandez-Fernandez *et al*.,

2002). This makes $K_{Ca}1.1$ an interesting player in many of the physiological processes that human bronchial epithelial cells participate in.

1.5.2 $K_{Ca}3.1$

The SK subfamily of K_{Ca} channels has four members (SK1-4) and of those members, SK4 or $K_{Ca}3.1$ is the only one that is restricted to nonneuronal tissues (Stocker, 2004). In contrast to $K_{Ca}1.1$ channels, there is no dependence on membrane potential in the biophysical characteristics of $K_{Ca}3.1$ and therefore its S2-S4 segments do not contribute to voltage sensing. Its gating kinetics depend entirely on changes in $[Ca^{2+}]_i$, and its channel topology is much more similar to K_v channels than $K_{Ca}1.1$ (Figure 2A) (Kohler *et al*., 1996; Berkefeld *et al*., 2006). It was originally hypothesized that there was a binding site for Ca^{2+} within the $K_{Ca}3.1$ channel; however, further investigation revealed that exogenous calmodulin (CaM) was instead the Ca^{2+} sensor related to this channel (Xia *et al*., 1998). Activators of $K_{Ca}3.1$ include 1-ethyl-2-benzimidazolinone (EBIO), while clotrimazole and TRAM-34 inhibit the channel (Devor *et al*., 1996; Ishii *et al*., 1997; Jensen *et al*., 1999). $K_{Ca}3.1$ plays an important role in regulating cell proliferation in T lymphocytes, since it controls the progression of these cells through the late G_1 phase of the cell cycle (Ghanshani *et al*., 2000). $K_{Ca}3.1$ also plays various roles in epithelial physiology, from Ca^{2+}-dependent ion transport in the airways (Devor *et al*., 1999; Cowley and Linsdell, 2002), to regulating cell volume in times of hyposmotic stress in tracheal epithelial cells (Vasquez *et al*., 2001). Despite the fact that $K_{Ca}3.1$ channels are becoming very widely studied, their roles in airways epithelial cells are still not completely clear.

1.6 K^+ channels in cell proliferation

As mentioned above, the epithelium also plays an important role in wound repair and cell proliferation in the airways. The respiratory system is always exposed to the external environment; therefore, there is an increased risk for epithelial injury if the MCC defense mechanism does not work properly. Respiratory diseases such as CF are especially prone to tissue damage due to increasing inflammation and epithelial wound repair must be implicated in order to keep the lungs healthy (Bardou *et al*., 2009).

Epithelial repair begins after the cell is exposed to injury by infection or inflammation. Neighbouring epithelial cells spread and de-differentiate and then migrate to cover the injured areas. Airway epithelial progenitor cells also migrate and proliferate in order to increase cell number. These cells then re-differentiate in order to function as airway epithelial cells (Bardou *et al*., 2009; Crosby and Waters, 2010). It has also been shown that after epithelial cells are injured those that survive release growth factors such as epidermal growth factor (EGF), which acts through EGF receptors (EGF-R), and mediate a mitogenic response important to migration and airway epithelial repair (Boxall *et al*., 2006; Trinh *et al*., 2007).

Not only is EGF an important component to the airway repair processes, but it has also been shown that K^+ channels are also important in alveolar and bronchial repair (Trinh *et al*., 2007; Trinh *et al*., 2008). In these studies it was found that when $K_v7.1$ and $K_{Ca}3.1$ were inhibited in bronchial epithelial cells, EGF-stimulated wound healing, cell migration and cell proliferation was reduced. It was also shown that CF bronchial epithelial cells took longer to heal in the presence of EGF than normal bronchial epithelial cells and this may be related to the expression of K^+ channels. $K_v7.5$ has also been reported to play a role in the proliferation of skeletal myoblast cells in rats, as it is up-regulated in myoblast growth and inhibition causes the arrest of myoblasts in the G_1 phase of the cell-cycle (Roura-Ferrer *et al*., 2008).

The relationship between K^+ channel activity and cell proliferation is still not completely understood, as it stems from previous studies looking at the aberrant or over-expression of K^+ channels in cancer and the dependence of cell-cycle progression to K^+ channels (Figure 3) (Wonderlin and Strobl, 1996; Bianchi *et al*., 1998; Meyer *et al*., 1999; Cherubini *et al*., 2000; Ouadid-Ahidouch *et al*., 2001; Smith *et al*., 2002; Wang *et al*., 2002). It has been concluded that in order to progress through the G_1 phase of the cell cycle, K^+ channels are required to hyperpolarize the membrane. Thus when $K_v10.1$ was inhibited in MCF-7 cells, it decreased proliferation and arrested the cell in G_1 (Wonderlin and Strobl, 1996; Ouadid-Ahidouch *et al*., 2001). It has also been shown that by silencing $K_v4.1$ channels through small interfering RNA (siRNA) targeting, proliferation in tumourigenic human mammary epithelial cells was significantly reduced (Jang *et al*., 2009). Since these channels are important in cancer epithelial cell pathophysiology and in

other normal cell types, perhaps there is a place for them in cell proliferation relating to normal and CF bronchial epithelial cell physiology, and further investigation is required to examine this theory.

1.7 K^+ channels in cell volume regulation

Epithelial cells are also involved in the regulation of cell volume, and changes in cell volume have been one of the main candidates as to how K^+ channel activity is related to cell proliferation, since cells change their volume as they progress through the cell cycle (Lang *et al*., 1998). Cells are also constantly exposed to altered intracellular and extracellular osmolarities, and they are able to maintain an optimum volume by activating various membrane proteins (Lang *et al*., 1998). Water movement parallels these changes in osmolarity, and contributes to the changes that occur in cell volume. In response to hyperosmotic stress, water will exit the cell via osmosis and through water-transporting proteins on the cell membrane, including aquaporins and the K^+/Cl^--co-transporter (KCC), due to the osmotic pressure gradient, (Zeuthen, 1994; Agre, 2006). Cells will then shrink in order to equilibrate the intracellular and extracellular osmolarities, and then attempt to return to their normal volume by activating $Na^+/ K^+/2Cl^-$ co-transporters, as well as Na^+/H^+ exchangers and in some cells, Na^+ channels. This is then followed by a gain of water and known as regulatory volume increase (RVI) (Lang *et al*., 1998; Mongin and Orlov, 2001). In contrast, when cells are exposed to hyposmotic stress, water will enter the cell and cause it to swell, activating K^+ and anion channels. This is then followed by the loss of water, which allows the cell to decrease back down in volume, known as regulatory volume decrease (RVD) (Lang *et al*., 1998). Hyposmotic changes to the external environment have been previously shown to affect human airway epithelial cells, and these cells responded by activating K_{Ca} channels, although different members of the K_v family also reportedly play a role in the RVD response (Vazquez *et al*., 2001; Fernandez-Fernandez *et al*., 2002; Harron *et al*., 2009). It has also been shown that $K_v7.1$ and $K_v11.1$ play an important role in regulating the RVD response in MCF-7 cells (vanTol *et al*., 2007; Roy *et al*., 2008). However, in Calu-3 cells it was shown that members of the K_v7 family other than $K_v7.1$, as well as $K_v4.1$ and $K_v4.3$ all contributed to

the RVD response (Harron *et al*., 2009). It is important to investigate which K^+ channels may play integral roles in mediating the RVD response in bronchial epithelial cells.

1.8 16HBE14o- and CFBE41o- cells as model cell lines

For this research project, both normal human bronchial epithelial cells, as well as those with a CF phenotype were used. The 16HBE14o- (HBE) cell line is a widely used model that was generated by Cozens and colleagues in 1994 by the transformation of normal human bronchial epithelial cells. These cells are widely used due to their easy maintenance and their avoidance of many problems that occur when using primary tissues, such as the high costs and reproducibility issues (Ehrhardt *et al*., 2002). HBE cells maintain the differentiation associated with native human bronchial epithelial cells, including the ability to become polarized and the presence of functional CFTR protein (Ehrhardt *et al*., 2002).

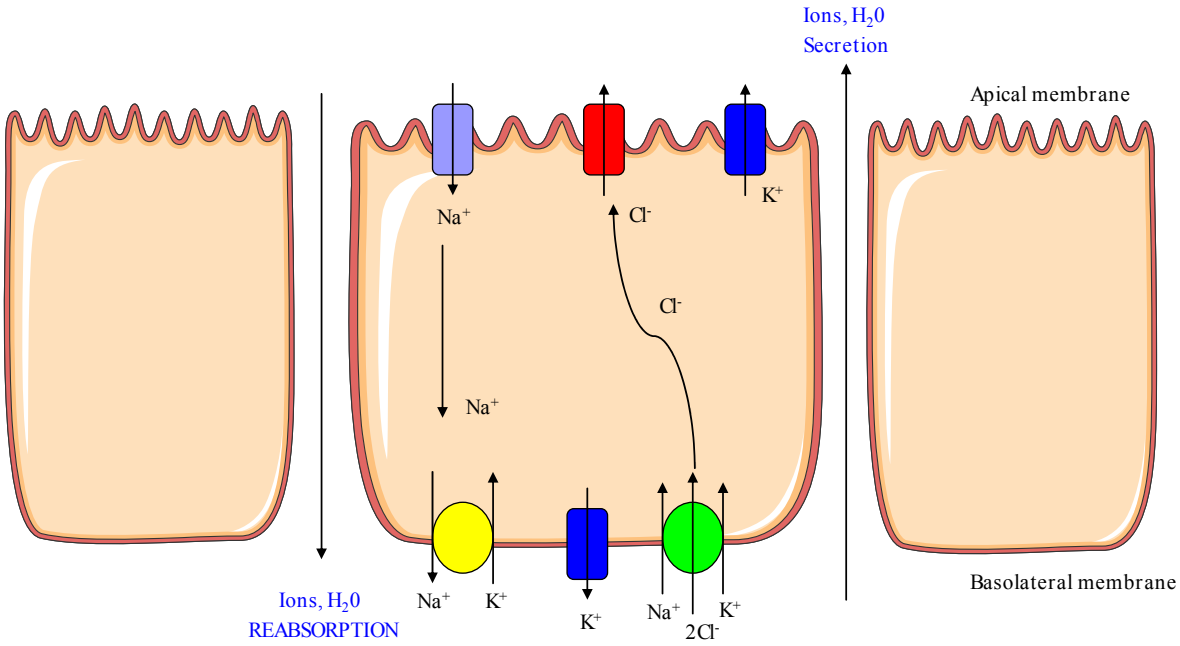
The CFBE41o- (CFBE) cell line is another widely used model that was generated by the transformation of CF bronchial epithelial cells. Not only are these cells homozygous for the ΔF508 mutation, but the mutation also stays intact over multiple passages (Ehrhardt *et al*., 2005). CFBE cells can also maintain the differention associated with human CF bronchial epithelial cell including the formation of cell layers that are not only electrically tight but also have cell-cell connections that are still functional (Ehrhardt *et al*., 2005). Although there are limitations with using cell lines over native cells (see Section 4.4.4), because HBE and CFBE cells maintain many of the same characteristics as their native cell counterparts, including the expression of particular K^+ channels (Namkung *et al*., 2009), they are good models for studying the different physiological functions of healthy and CF human bronchial epithelial cells.

1.9 Study Objectives

The basis of this project is to investigate the physiological role that various K_v and K_{Ca} channels play in human bronchial epithelial cells. Since these channels have been shown to play important roles in cell proliferation and cell volume regulation in other cell lines, it is of interest to delineate their functions in bronchial epithelial cells that are both healthy, as well as those representing the CF phenotype. Based on the evidence, I

hypothesize that K_v7.1, 7.5, 4.1, 4.2, 4.3, 10.1 and 11.1, and K_{Ca}1.1 and 3.1 play an important role in cell proliferation and regulation of cell volume under hyposmotic stress and that these findings could give some insight towards finding a cure for the devastating disease that is CF.

A HEALTHY INDIVIDUALS



B CF INDIVIDUALS

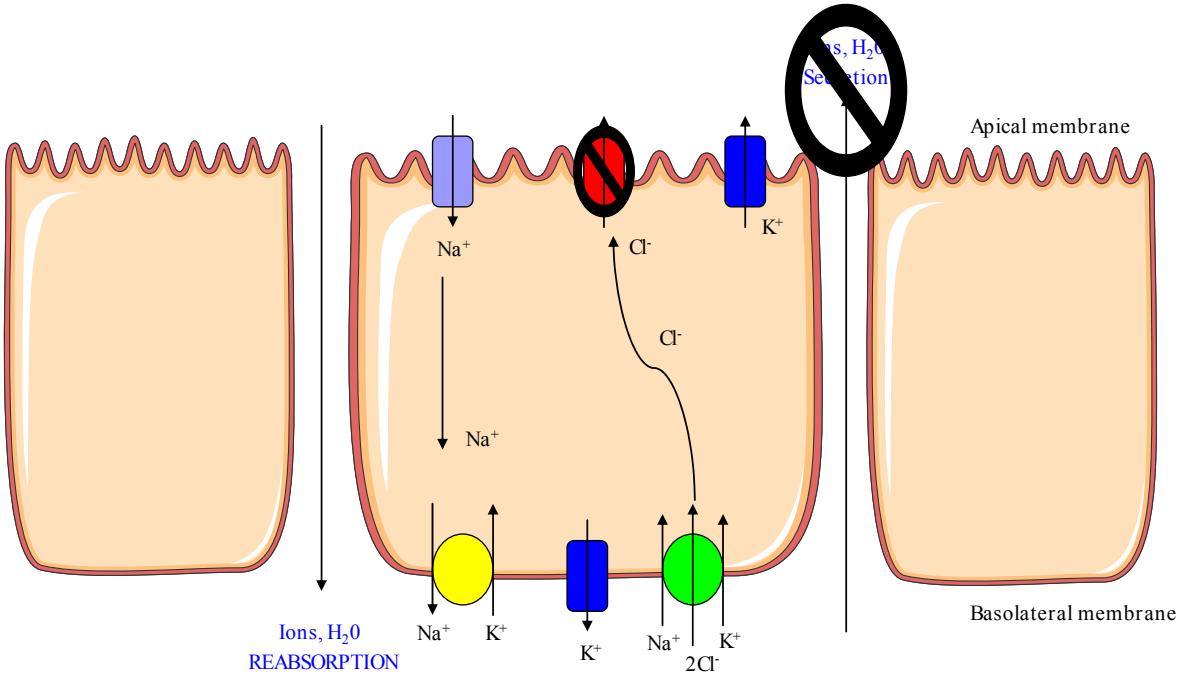


Figure 1. Representative model of ion transport in airway epithelial cells. In order to maintain an adequate PCL volume there must be a balance between Na^+ absorption and Cl^- secretion across the epithelial cells that line the airways. (A) The Na^+/K^+-ATPase on the basolateral membrane sets up the electrochemical gradient of the cell. As it actively pumps 3 Na^+ ions out of the cell, it pumps 2 K^+ ions in. These K^+ ions are recycled through basolateral and potentially apical K^+ channels. The decrease in $[Na^+]_i$ inside the cell creates the driving force for the entry of Na^+ through apically located ENaC. This Na^+ is then expelled via the Na^+/K^+-ATPase pump. As Na^+ ions undergo transepithelial transport, water and other ions osmotically follow this movement via the paracellular pathway. The $Na^+/2Cl^-/K^+$ co-transporter on the basolateral membrane then transports Na^+, $2Cl^-$ and K^+ ions into the cell, and Na^+ exits via the Na^+/K^+-ATPase pump as K^+ is again recycled by K^+ channels on the basolateral and apical membranes. Cl^- undergoes transepithelial transport and is secreted from apical Cl^- channels, including CFTR. Once again, water and other ions osmotically follow this Cl^- movement via the paracellular pathway. (B) In people who suffer from CF, the dysfunction in their CFTR protein causes a decrease in Cl^- and other anion secretion. This decreases the paracellular transport of water to the lumen and creates dehydrated mucus that causes an array of complications in the airways (Bardou *et al*., 2008).

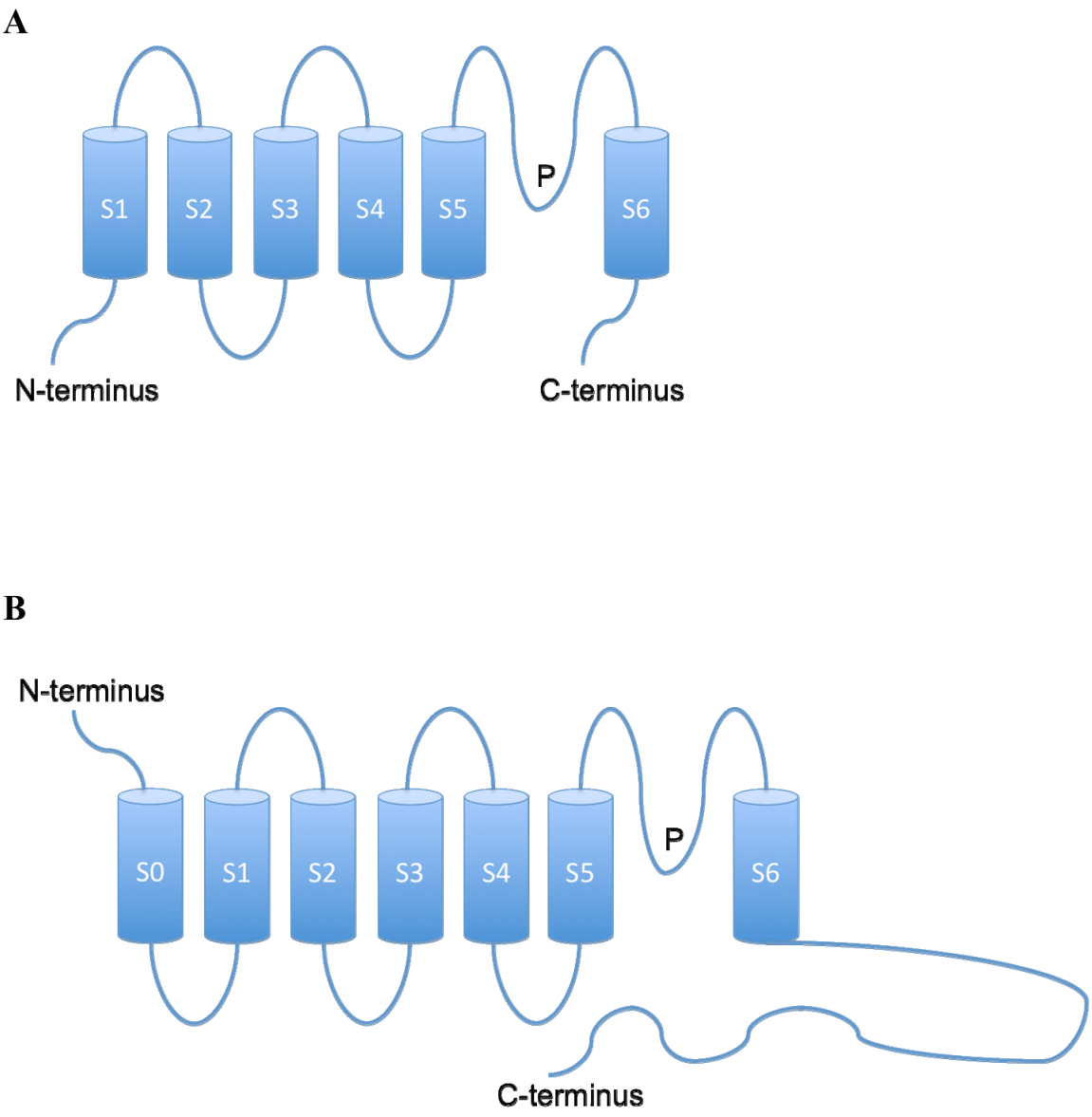


Figure 2. Structural model of various types of K^+ channels. (A) Represents K_v channel topology, with 6 TMDs (S1-S6), the pore loop between S5 and S6 (P) and the intracellular NH_2 and COOH termini, shown by the N- and C- terminus respectively. (B) Represents the $K_{Ca}1.1$ channel topology, with 7 TMDs (S0-S6), the pore loop between S5 and S6 (P) and the extracellular NH_2 terminus and longer intracellular COOH terminus (N- and C- terminus respectively) (Bardou *et al*., 2008; Berkefeld *et al*., 2010).

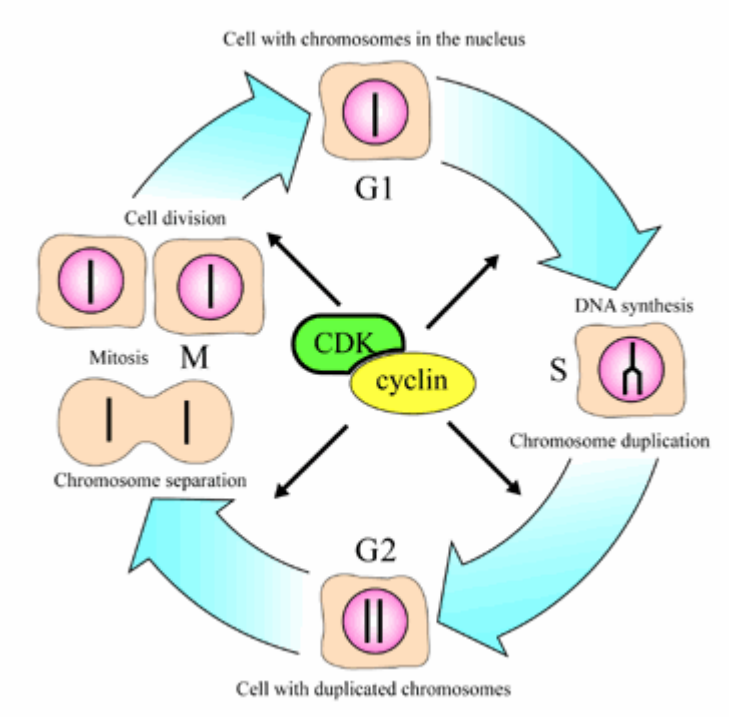


Figure 3. Schematic representation of the cell cycle. G_1, S, G_2 and M represent the first gap, DNA synthesis, second gap and mitotic phases of the cell cycle respectively. The cell grows in the G_1 and when it becomes large enough it enters the S phase. The S phase consists of DNA synthesis and chromosomes duplicate. During the G_2 phase, the cell continues to grow and prepares for cell division, which occurs during the M phase and creates two daughter cells. K^+ channels play a role in the cell's progression through various phases of the cell cycle, along with various cyclins and cyclin-dependent kinases (CDK) (Hartwell *et al*., 2001).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture

16HBE14o- (HBE) human bronchial epithelial cells and CFBE41o- (CFBE) cystic fibrosis human bronchial epithelial cells were obtained from Dr. Dieter Gruenert (California Pacific Medical Center, San Francisco, CA, USA), and were originally immortalized through transformation with the plasmid pSVori$^-$ (Cozens *et al*., 1992; Cozens *et al*., 1994). Both cell lines were maintained in a humidified incubator at 37°C with 5% CO_2. Stock cells, which were passaged each week, and grown in Minimum Essential Media (MEM) containing phenol red, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin (all from Invitrogen, Burlington, ON). Medium was replaced every 2-3 days from stock cells that were grown in 75 cm^2 polystyrene tissue culture flasks (Corning, Lowell, MA, USA) and coated with 1.5 ml of a fibronectin coating solution (1-5 $\mu g/cm^2$) for 3 hr before use (Invitrogen; Sigma-Aldrich, Oakville, ON; BD Biosciences, Franklin Lakes, New Jersey, USA) (Appendix C), as described in Ehrhardt *et al*., 2002. All experiments were performed between passages 8-20 and occurred when the cells reached between 90-100% confluency.

To passage the cells, the media was removed and they were washed with 10 ml of RNase-free 1X phosphate buffered saline (PBS) with 1.1 mM glucose and 684 μM EDTA (1XPBS GE) (Appendix C). Cells were detached from the surface of the flask using 1 ml of 0.25% trypsin (Invitrogen) and incubated at 37°C for 20-30 min. After the cells were completely detached they were re-suspended in 6 ml of media, counted using a Coulter Counter (see below) and from this solution 4,000,000 HBE and 500,000 CFBE cells were put into the stock flasks in order to continue the cell line. HBE cells were smaller than CFBE cells and therefore more cells were required for the stock flasks to become confluent (HBE= 15.74 ± 0.054 μm; CFBE= 18.64 ± 0.19 μm; n=17). For each individual experiment the number of cells, how they were cultured and their treatment protocol is described below.

2.2 Cell Counting

Using a Coulter Counter, cell number was measured as a means to assess cell proliferation during the cell cycle on HBE and CFBE cells as follows. Approximately 200,000 HBE and 64,000 CFBE cells were put into each well of a six-well polystyrene tissue culture plate (BD Biosciences) coated with fibronectin (see above). The cells were incubated at 37°C in 10% FBS MEM for 24 hr, allowing them to attach to the plates. Cells were then treated in 1% FBS MEM with specific drugs at differing concentrations and durations, as described in Table 2. After 48 hr in the presence of a drug or vehicle control, the media was removed and each well was washed with 1XPBS GE. 1 ml of 0.25% trypsin was added to each well and the plates were incubated at 37°C for 10 min, or until the cells were completely detached. A Coulter Counter (Beckman Coulter Canada, Inc., Mississauga, ON) was used to count the number of cells suspended in each sample. 1 ml of each sample was added to 9 ml of 1XPBS GE, mixed by pipetting, and placed into the Coulter Counter. Briefly, as cells flow into the aperture, they increase the resistance between the electrodes, and therefore increase the voltage as well. Each cell that passes through the aperture records a voltage pulse, and the number of pulses measured equals the number of cells in a sample, whereas the amplitude of the pulse is proportional to the cell volume (Beckman Coulter, Inc., 2011).

2.3 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

In order to measure the potential cell death in response to drugs, LDH cytotoxicity assays (Caymen Chemical, Michigan, USA) were performed. LDH is a soluble enzyme located in the cytosol of cells, and released into the surrounding culture medium upon cell damage or lysis, and is commonly used as an indicator of cell cytotoxicity (Haslam *et al*., 2000; Wolterbeek and van der Meer, 2005). Either 50,000 HBE or 20,000 CFBE cells were plated in 24-well polystyrene tissue culture plates coated with fibronectin (BD Biosciences) and grown for 24 hr. The next day they were treated with specific drugs at differing concentrations (Table 2) and returned to the incubator for 48 hr. 500 µl of medium from each well was transferred to 1.5 ml sterile microcentrifuge tubes, centrifuged at 400 X g relative centrifugal force (RCF) for 5 min. 100 µl of each supernatant was transferred to corresponding wells in a 96-well polystyrene tissue culture

plate (BD Biosciences). 100 µl of the reaction solution was added to each well, which consisted of 100X of each NAD^+, lactic acid, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT), LDH diaphorase and finally enough assay buffer to bring the reaction solution up to the amount needed to add 100 µl to each well (eg. 17 wells = 1700 µl reaction solution). The 96-well plate was incubated for 30 min at room temperature with gentle shaking and then the absorbance was read at 490 nm on a microplate reader (Beckman Coulter Canada, Inc.).

2.4 RNA Extraction

In order to detect specific K^+ channel mRNA expression in HBE and CFBE cells, total RNA was extracted and reverse transcription-polymerase chain reaction (RT-PCR) was performed. As a first step either 200,000 HBE or 64,000 CFBE cells were cultured in 6-well plates coated with fibronectin and grown to approximately 90-100% confluency in order to optimize the amount of RNA extracted. After removing the media each well was washed with 2 ml of 1XPBS GE for 30 s and 1 ml of Trizol Reagent™ (Invitrogen) was added to each well. Cells were then incubated at room temperature for 5 min and then homogenized by pipetting. Each sample was transferred to a 1.5 ml sterile micro-centrifuge tube and 200 µl of chloroform (Fisher Scientific, Ottawa, ON) was added. The micro-centrifuge tubes were vortexed for 15 s, incubated at room temperature for 3 min and centrifuged at 4°C for 15 min at 13,000 revolutions per minute (RPM). 400 µl of the top colourless aqueous phase containing RNA was removed and transferred into new 1.5 ml sterile micro-centrifuge tubes and 500 µl of isopropanol (Sigma-Aldrich) was added. The tubes were then vortexed, incubated at room temperature for 10 min and centrifuged at 4°C for 10 min at 13,000 rpm. The supernatant was removed and the tubes were centrifuged at room temperature to bring down any residual RNA. This RNA pellet was then washed with cold 75% ethanol. The tubes were vortexed and then centrifuged at 4°C for 5 min at 13,000 rpm and the wash step was repeated. Finally, the supernatant was removed, and with the lids of the tubes left open they were placed in the water bath at 55-60°C to allow the ethanol to evaporate. After this, 20 µl of autoclaved water was added to each tube and they were stored at -80°C.

The concentration and purity of the extracted RNA was measured using a GeneQuant Pro Spectrophotometer (Biochrom, Cambridge, U.K.). 1 µl of each sample was added to 69 µl of sterile water in 0.5 ml micro-centrifuge tubes and vortexed. The RNA sample was then added to a cuvette and inserted into the spectrophotometer, and the RNA concentration (in µg/µl) was recorded. The purity of the RNA was measured and recorded as the 260/280 ratio and a value of > 1.8 indicated a sample of nucleic acid with minimal protein contamination.

2.5 Reverse Transcription of RNA to Single Stranded cDNA

Complimentary deoxyribonucleic acid (cDNA) was formed by RT of the RNA samples. 2 µg of RNA was added to 1 µl of 500 µg/ml oligoDT (Promega, Madison, WI) and sterile water was added to reach a final volume of 15 µl. Samples were heated to 70°C for 3 min and then cooled to 4°C for 2 min. 2 µl of 1st strand cDNA buffer, 2 µl of 5mM deoxyribonucleoside triphosphate (dNTP) and 1 µl of 200 U/µl Moloney Murine Leukemia Virus (M-MLV) RT (all Invitrogen) were added to each sample and mixed by pipetting. A negative reverse transcription was performed as a control step, which consisted of 1 µl of sterile water instead of the M-MLV RT. The samples were incubated at 42°C for 1 hr and then heated to 80°C for 5 min in order to inactivate the reverse transcription enzyme. 80 µl of sterile water was added to each sample once the reaction was complete to bring the final volume up to 100 µl and the samples were stored at -20°C.

2.6 Polymerase Chain Reaction (PCR)

The specific gene fragments for the K^+ channels of interest were amplified using PCR. For each reaction, each sample contained 2 µl of cDNA, 15.5 µl sterile water, 1.5 µl of 25mM $MgCl_2$ (Fermentas, Burlington, ON), 2.5 µl of 10X Taq Buffer (Fermentas), 1 µl of 5mM dNTP, 1 µl of sense primer and 1 µl of antisense primer (Invitrogen) and 0.5 µl of Taq DNA Polymerase (Fermentas). Primer information, annealing temperatures and references are described in detail in Table 3. The conditions were optimized for each cell line and the working concentrations of each primer was 10 µM. Sterile water was substituted for cDNA in the negative control experiments and the housekeeping gene

HPRT was used in positive control experiments to test the integrity of the cDNA. cDNA from another airway epithelial cell line, Calu-3 was used as a positive control for certain genes.

2.7 Agarose Gel Electrophoresis

PCR products were viewed by agarose gel electrophoresis. A 1.5% agarose gel was made by adding 50 ml of 1XTris/Borate/EDTA (TBE) buffer (Appendix C) to 0.75 g of agarose (Fisher Scientific) and microwaving for 2 min. 2.5 µl of 0.5 µg/µl ethidium bromide (Sigma-Aldrich) was added to the mixture, which was then poured into the gel box and allowed to set for 20 min. 5 µl of a 100 bp DNA Ladder (Fermentas) was added to the first lane in the gel and then 15 µl of each PCR reaction, which had been mixed with 5 µl of 6X loading dye (Fermentas), was added to the next lanes. The voltage was set to 90 volts (V) and the gel was run for 45 min. A UV Transiluminator UVP BioDoc-It system (Upland, CA, USA) was used to view the amplicons.

2.8 Western Blotting

Western blotting was performed to detect protein expression in both cell lines. Both HBE and CFBE cells were grown in 100 X 20 mm polystyrene tissue culture dishes (BD Biosciences) to 90-100% confluency in order to optimize the amount of protein extracted, and total cellular protein was extracted. Cells were rinsed twice with 10 ml of cold 1XPBS GE and then 500 µl of RIPA buffer (Appendix C), with 5 µl HALT^{TM} protease-inhibitor cocktail (Fisher Scientific) and 2 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) was added to the cells and they were left on ice for 30 min. The cells were then detached from the dishes using cell scrapers (Sarstedt Inc, Newton, NC, USA) and placed in a 1.5 ml micro-centrifuge tube and centrifuged at 13,000 RPM for 30 min at 4°C. The supernatant was then analyzed using the Bradford method (Bio-Rad, Hercules, CA, USA) and a microplate reader to measure the protein concentration in each sample prior to storage at -20°C. 10-60 µg of HBE or CFBE protein was used per lane for Western blot analysis and diluted with 2X Laemelli loading buffer (Bio-Rad) with 5% ($^{v}/_{v}$) β-mercaptoethanol and heated in a water bath for 3 min at 95°C. Sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis gels were used to separate the proteins

and run for 1.5 hr in 1X running buffer (Appendix C). These gels were composed of a stacking gel (5%) and a resolving gel (7.5%) (Appendix C). First, the protein migrated down the stacking gel at 70 V and the voltage was then increased to 100 V when the protein reached the resolving gel. Once the protein had migrated down the resolving gel, the stacking gel was removed and the protein was transferred onto a nitro-cellulose membrane at 100 V for 1 hr in transfer buffer on ice (Appendix C). Membranes were then blocked for 1 hr in room temperature with 5% skim milk powder in Tris-base saline (TBS) containing 0.1% Tween 20 (Bio-Rad) (TBS-T) (Appendix C). The membranes were incubated overnight at 4°C with gentle shaking, with specific primary antibodies in milk (Sigma-Aldrich). After primary antibody incubation, membranes were washed 3 times for 15 min with TBS-T and once for 15 min with TBS. Membranes were then incubated for 2 hr at room temperature and shaken gently with the appropriate secondary antibody in milk. The specific primary and secondary antibody information is described in Table 4. After secondary antibody incubation each membrane was once again washed 3 times for 15 min in TBS-T and once for 15 min in TBS. In order to visualize protein band expression on the nitro-cellulose membrane, the Amersham Enhanced Chemiluminesence Plus Western blotting detection system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used following the manufacturers' protocol.

2.9 Cell Volume Regulation

Experiments were performed to measure changes in cell volume under conditions of altered osmolarities following protocols designed by vanTol and colleagues in 2007, and Harron and colleagues in 2009. Briefly, single HBE or CFBE cells were plated on glass coverslips in 35 X 10 mm polystyrene tissue culture dishes (BD Biosciences) and incubated between 3 to 7 hr to allow the cells to attach to coverslips. Bath solutions are described in Appendix 1. The osmolarities of the bath solutions were measured using a vapour pressure osmometer (Wescor, Logan, UT, USA) and the respective osmolarities were 124.2 ± 0.49 mOsM, 180.3 ± 0.46 mOsM, 206.6 ± 0.52 mOsM, 259.5 ± 0.70 mOsM, 327.3 ± 0.46 mOsM, 482.3 ± 0.41 mOsM and 528.5 ± 0.43 mOsM (n=24). The glass coverslips were first placed on the microscope, and solutions were continuously perfused at a rate of 1 ml/min using a peristaltic pump (Gilson, Middleton, WI, USA).

Cells were equilibrated in isosmotic (327 mOsM) solution for 5 min and then switched to a hyperosmotic or hyposmotic solution containing either vehicle or drug for 25 min. At the end of the experiment, cells were returned to isosmotic conditions for 5 min in order to ensure their viability and ability to return to their original size. For experiments involving K^+ channel inhibitors and RVD, the hyposmotic solution measuring 180 mOsM was used, since it had the greatest effect on the RVD response (Figure 8). Cells were visualized using a Zeiss Axioskop FS microscope (Oberkochen, Germany) and a water-immersion 63X objective lens. Every minute, images of the cells were captured by a PixelLINK 1394 camera with accompanying Capture SE version 4.1 software (PixelLINK, Ottawa, Canada) and cell area was analyzed with ImageJ version 1.36b software (National Institute of Health, Bethesda, MD, USA). Assuming a spherical shape, cellular radius (R) and volume (V) were calculated from the equations $R = \sqrt{(area/\pi)}$ and $V = 4/3(r\pi^3)$, respectively, and cell volume was normalized to that at t = 0 (vanTol *et al*., 2007). Results for RVD experiments were presented as percentage of RVD (% RVD) ± standard error (SE). % RVD was calculated as $100(1-[(V_{25min}-1)/(V_{max}-1)])$, where V_{max} is the peak volume in the hyposmotic solution. % RVI was calculated as $100(1-[(V_{25min}-1)/(V_{min}-1)])$, where V_{min} is the minimum volume in hyperosmotic solution (Harron *et al*., 2009).

2.10 siRNA Transfection

HBE cells were transfected with $K_v7.1$ and $K_v7.5$ siRNA duplexes using siTran 1.0 Transfection Reagent, tagged with Trilencer-27 TYE-563 labeled fluorescent transfection control siRNA duplex (1 nmol) (all from OriGene Technologies, Rockville, MD, USA). Three different gene specific 27mer siRNA duplexes (2 nmol per vial) for each K^+ channel were provided, as well as a universal scrambled negative control siRNA duplex (2 nmol) and an RNAse free siRNA duplex re-suspension buffer. Each siRNA duplex was re-suspended in 100 µl RNAse free duplex buffer to produce a 20 µM final concentration, while the fluorescent transfection control siRNA duplex was re-suspended in 100 µl RNAse free duplex buffer to produce a 10 µM final concentration. Duplexes were then heated to 94°C for 2 min, cooled at room temperature and stored at -20°C until used. Experiments were performed on cells in 6-well plates when they reached 60%

confluency. Briefly, 5 µl of siRNA was mixed with 140 Opti-MEM® Reduced Serum Media (opti-MEM) (Invitrogen) and incubated at room temperature for 5 min. In parallel, 20 µl siTrans reagent and 115 µl opti-MEM medium were combined and incubated for 5 min. The siRNA/Opti-MEM mixture was then added to the siTrans/Opti-MEM mixture and incubated for 10 min. While incubating, cells were washed with 1 ml 1XPBS GE for 30 s. At the end of the incubation period, the siRNA/siTrans mixture was pipetted onto the cells and 2.5 ml of MEM (without FBS) media was placed on top. Next the cells incubated for 24 hr and were then switched to 5% FBS MEM medium. Protein extraction was performed 48 hr after the transfection and analyzed by Western blot.

2.11 Plasmid DNA Transfection

In order to suppress the expression of $K_v7.1$ in HBE cells, site-directed mutagenesis that had been previously performed in our lab was used to create a NH_2-terminal truncated isoform of $K_v7.1$ that acts as a dominant negative mutation known as ΔN- $K_v7.1$ (Jiang *et al*., 1997; vanTol *et al*., 2007). Bacterial stocks for this mutation were grown up and DNA was extracted following the QIAprep Spin Midiprep Kit protocol (QIAGEN Inc., Toronto, ON). The concentration of the extracted DNA was measured (in ng/µl) using a GeneQuant Pro Spectrophotometer (see 2.4 RNA Extraction). HBE cells were plated in 60 X 15 mm polystyrene tissue culture dishes (BD Biosciences) and grown in 10% FBS MEM (without antibiotic) media until they reached 60% confluency. These cells were then transfected with pIRES2-EGFP-ΔN- $K_v7.1$ plasmid DNA. Briefly, 3.33 µl of DNA (600 ng/ul) was added to 100 µl of opti-MEM media in one tube, while 6 µl lipofectamine 2000 (Invitrogen) was added to 100 µl of opti-MEM media in another tube. Both mixtures were left to incubate at room temperature for 5 min, and then added together and left to incubate for 20 min. This mixture was then added to the cells after the existing media was removed, followed by 1.5 ml of serum-free and antibiotic-free MEM media. The cells were incubated at 37°C for 5 hr and then switched to 10% FBS MEM media and allowed to grow in the incubator. 48 hr after transfection, the cells were washed with 1 ml PBS GE and 200 µl trypsin was added to each plate for 10 min at 37°C in order to detach them. The cells were resuspended in 1 ml of 10% FBS MEM media and seeded on glass coverslips in 35

X 10 mm polystyrene tissue culture dishes and incubated for 3 to 7 hr before use in order for cells to attach to the coverslips. Cell volume experiments were then performed (see Section 2.9). Control experiments were performed by transfecting the cells with the pIRES2-EGFP vector alone, and followed the same protocol as above.

2.12 Drugs

Table 2 outlines the drugs used, the channels they inhibit, dilution vehicles, stock concentrations, half maximal inhibitory concentration (IC_{50}) values and their sources. Various concentration-response curves were performed in order to test the minimal and optimal concentrations of the drugs and inhibitors were also tested for their cytotoxicity using LDH assays as described above.

2.13 Statistics

For cell counting experiments, cell volume regulation, and LDH assays data was analyzed using ANOVA with Tukey's post hoc tests, followed by a Students t-test for unpaired values. A P-value of < 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 HBE and CFBE cells express K_v and K_{Ca} channel mRNA.

RNA extraction and RT-PCR were performed on HBE and CFBE cells in order to investigate mRNA expression for various K^+ channels. The PCR transcripts for K_v7.1 (Figure 4A), K_v7.5 (Figure 4B), K_v4.1 (Figure 4C), K_v4.2 (Figure 4D), Kv11.1 (Figure 4F), K_{Ca}1.1 (Figure 4G) and K_{Ca}3.1 (Figure 4H) were all detected for HBE (H) and CFBE (CF) cells at the expected amplicon size (arrow). The PCR transcript for Kv10.1 (Figure 4E) was detected in CFBE cells at the expected size, but not in HBE cells. After repeated attempts using different PCR conditions no transcript was detected for K_v4.3 (Figure 4I) in HBE or CFBE cells; however it was detected in Calu-3 (C) cells at the expected amplicon size. The negative RT samples (-), which consisted of H_2O alone, did not detect any of the PCR transcripts (Figure 4A-I). All PCR experiments were run for 40 cycles and performed at least 3 times.

3.2 HBE and CFBE cells express K_v7.1 and K_v7.5 protein.

Protein extraction and Western blots were performed on HBE and CFBE cells in order to investigate K_v7.1 and K_v7.5 protein expression. Protein was detected for K_v7.1 at 75 kDa in HBE (+H) and CFBE (+CF) cells; however, when the primary antibody was omitted (-), no protein was detected (Figure 5A). This acted as a negative control, because there was no commercially available blocking peptide for this antibody. Protein was also detected for K_v7.5 at 116 kDa in both cell lines, and again no protein was detected when the primary antibody was omitted due to the lack of commercially available K_v7.5 blocking peptide (Figure 5B). Each experiment was performed at least 3 times.

3.3 Various K_v and K_{Ca} channel inhibitors affect cell proliferation in HBE and CFBE cells.

A Coulter Counter was used to measure cell number as a means to assess the contribution of various K^+ channels to cell proliferation in these model cell lines. First, the doubling time of these cells were investigated in order to measure how long it took

before the population of cells entered the M phase of the cell cycle. This is important, since K^+ channels play a role in allowing the cell to progress through the G_1 phase of the cell cycle; therefore, by treating the cells with K^+ channel inhibitors before they divide, they are able to become partially synchronized in this phase (Wonderlin and Strobl, 1996). It must be noted however, that when the doubling time has been reached, it does not necessary mean that all the cells have divided, as some cells of the population may be in other phases as well. Doubling time experiments were performed by plating 200,000 HBE and 64,000 CFBE cells in 6-well plates and letting them grow over 96 hr. They were counted every 24 hr and the log of the cell number was plotted. A line of best fit was calculated, and the slope of that line was used to determine the doubling time (NIH Chemical Genomic Center, 2009). For HBE cells, the doubling time was 29.7 hr and for CFBE cells the doubling time was 31.6 hr (Figure 6). Therefore, for all further cell proliferation experiments, cells were treated with inhibitors 24 hr after they were plated to allow the inhibitors to act before the cells divided and counted 48 hr later.

The drugs used to inhibit the channels of interest are described in Table 2 along with the corresponding channels they act on. Inhibitor concentrations were chosen from previous literature (Roy *et al*., 2010; Harron *et al*., 2009) and concentration-response experiments (not shown). HBE cell proliferation was significantly inhibited by XE991, flecainide and TRAM-34 (Figure 7A). XE991 (100 μM) inhibited cell proliferation by 20 ± 4.1% ($P < 0.05$), flecainide (100 μM) inhibited cell proliferation by 37 ± 6.3% ($P < 0.05$) and TRAM-34 (10 μM) inhibited cell proliferation by 27.4 ± 5.4% ($P < 0.05$), all compared to controls. Chromanol 293B (100 μM), stromatoxin (20 nM), astemizole (3 μM), E-4031 (3 μM) and paxilline (2 μM) had no significant effect on cell proliferation in HBE cells ($P > 0.05$). CFBE cell proliferation was significantly inhibited by XE991, flecainide, astemizole and TRAM-34 (Figure 7B). XE991 (100 μM) inhibited cell proliferation by 38.2 ± 7.9% ($P < 0.05$), while flecainide (100 μM) inhibited cell proliferation by 55.3 ± 5.9% ($P < 0.05$), astemizole (3 μM) inhibited cell proliferation by 31.6 ± 6.0% ($P < 0.05$) and TRAM-34 (10 μM) inhibited cell proliferation by 27.2 ± 9.1% ($P < 0.05$), all compared to controls. 293B (100 μM), stromatoxin (20 nM), E-4031 (3 μM) and paxilline (2 μM) had no significant effect on cell proliferation in CFBE cells ($P > 0.05$). These results suggest that in both cell lines, members of the K_v7 family other

than K_v7.1 are involved in cell proliferation, since XE991 which inhibits K_v7.1-7.5 significantly inhibited cell number, and 293B which specifically inhibits K_v7.1 did not. It also suggests that K_v4.1 is involved in cell proliferation in both cell lines since flecainide which inhibits K_v4.1-4.3 significantly inhibits cell number and stromatoxin which specifically inhibits K_v4.2 does not. Since K_v4.3 channels are not expressed in these cell lines (Figure 4I) flecainide must presumably be acting on K_v4.1. K_{Ca}3.1 also plays a role in cell proliferation, since TRAM-34 significantly inhibits cell number in both cell lines. K_v10.1 plays a role in cell proliferation in CFBE cells only, since astemizole significantly inhibited cell number. These results concur with our previous findings that HBE cells do not express K_v10.1 mRNA (Figure 4E).

In order to confirm that decreases in cell number were not due to cell death, cell cytotoxicity assays were performed. LDH assays were performed on cells 48 hr following treatment with the drugs that produced a significant decrease in cell number (as seen in Figure 7). Neither application of 100 μM XE991, 100 μM flecainide, nor 10 μM TRAM-34 caused significant cell death in HBE cells (Figure 8A). Similarly, there was no cytotoxicity in CFBE cells following the application of 100 μM XE991, 3 μM astemizole, or 10 μM TRAM-34 (Figure 8B). However, significant cell death was seen with 100 μM flecainide. Lower concentrations of flecainide did not inhibit cell proliferation (not shown); therefore, it is likely that the decrease in cell number was due to cytotoxicity rather than a true effect on proliferation and it is unlikely that K_v4.1 plays a role in proliferation in CFBE cells (see Section 4.2.2). HBE cells did not experience these cytotoxic effects from 100 μM flecainide, and perhaps this is because HBE cells are more robust than their CF counterparts, and less easily killed.

3.4 Cell proliferation is not affected by the addition of the potential mitogens EGF, IGF-1 or E2.

The above experiments investigated the role of K^+ channels in basal cell proliferation. We were next interested in identifying the role of these channels in mitogenic-stimulated proliferation in HBE and CFBE cells. It has been reported that exogenous EGF (0.5 ng/ml) is required for cell migration and wound healing in both normal and CF bronchial epithelial cell lines, NuLi-1 and CuFi-1, respectively (Trinh *et al*., 2008); therefore, perhaps it is also important for cell proliferation in HBE and CFBE

cells. However, when cells were treated with increasing concentrations of EGF (0.1, 0.5, or 1 ng/ml for 48 hr), there was no significant increase in cell number (Figure 9A). It has also been reported that insulin-like growth factor-1 (IGF-1), is a powerful mitogen that is known to regulate the cell cycle in many cell systems (Pluteanu and Cribbs, 2011). However, when IGF-1 was applied to HBE and CFBE cells (0.1, 0.5, or 1 ng/ml for 48 hr), cell number did not increase (Figure 9B). Finally, 17β-estradiol (E2) was investigated, as this has been shown to cause the mammary epithelial cancer cell line MCF-7 to proliferate faster than controls (Brooks and Skafar, 2004). However, when HBE and CFBE cells were treated with E2 (0.01, 0.1, and 1 nM for 48 hr), it did not increase cell number (Figure 9C). Therefore, HBE and CFBE cells do not respond to the mitogenic-stimulation of EGF, IGF-1 or E2 and the role of K^+ channels in mitogen-stimulated versus basal cell proliferation could not be examined.

3.5 Cell proliferation is not affected by the addition of the potential K_v7 channel activator flupirtine maleate.

I was also interested in potentially identifying K^+ channel activators in order to investigate their role in cell proliferation. Flupirtine maleate has been proposed as a $K_v7.2$-7.5 activator involved in smooth muscle cells (Yeung *et al*., 2007); therefore, I hypothesized that the addition of flupirtine maleate to HBE and CFBE cells would increase cell number and in turn increase cell proliferation, due to the activation of K^+ channels involved in the cell cycle. When treated with flupirtine maleate (0.5, 1, and 5 μM for 48 hr) cell number did not increase (Figure 10). Therefore, perhaps flupirtine maleate does not activate K_v7 channels in bronchial epithelial cells or activation of these channels does not affect cell proliferation.

3.6 HBE cells exhibit an RVD response when exposed to hyposmotic solutions.

Cell volume regulation experiments were performed in order to investigate the K^+ channels involved in the RVD response in HBE cells. Initially, cells were placed in an isosmotic solution that measured 327.3 ± 0.46 mOsM. When HBE cells were switched to a more hyposmotic solution of 180.3 ± 0.46 mOsM, the cells increased in size over the first few minutes, and then reduced their volume towards their initial size. This decrease is known as the RVD response and measured 22.9 ± 0.74% when cell size was

normalized to fold change in volume (Figure 11A). When the osmolarity was changed to 206.6 ± 0.52 mOsM in order to represent a less severe hyposmotic shock, the cells increased in size but not to the same extent as in (A) and they exhibited an RVD response of only 4.72 ± 0.95% (Figure 11B). Finally, when the osmolarity was switched to 259.5 ± 0.70 mOsM to represent an even less severe hyposmotic shock, the cells showed a much smaller increase in size over the first few minutes and again only exhibited an RVD response of 3.31 ± 1.65% (Figure 11C). Therefore, all subsequent experiments pertaining to RVD were performed in hyposmotic solutions of 180.3 ± 0.46 mOsM in order to allow the cells to achieve an RVD response that was measurable and reproducible.

3.7 Various pharmacological K_v and K_{Ca} channel inhibitors affect the RVD response in HBE cells.

In order to investigate the contribution of K^+ channels to the RVD response, inhibitors were added to the hyposmotic solution and the % RVD was measured. The fold change in volume curves for selected inhibitors are shown in Figure 12, while Figure 13 shows a summary of these results. Cells were initially bathed in isosmotic solution (327.3 ± 0.46 mOsM) and were then switched to hyposmotic solutions (180.3 ± 0.46 mOsM) containing an inhibitor. Inhibitor concentrations were initially chosen from previous literature (Harron *et al*., 2009), however after comparing these concentrations with each drug's IC_{50} values, experiments were re-done at these IC_{50} concentrations (Table 2). Results between the two attempts did not vary (not shown); therefore, the lower (IC_{50}) values were used for all further experiments. When HBE cells were exposed to XE991 (0.75 μM), which inhibits K_v7 channel members, the RVD response decreased from 22.9 ± 0.74% to 5.6 ± 0.9%, exhibiting a significant 17.3% decrease in RVD ($P < 0.05$) (Figure 12A and Figure 13). In the presence of 293B (5 μM), which inhibits $K_v7.1$, the RVD response decreased from 22.9 ± 0.74% to 5.4 ± 1.2%, exhibiting a significant 17.5% decrease in RVD ($P < 0.05$) (Figure 13). In the presence of flecainide (50 μM), which inhibits K_v4 channel members, the RVD response decreased from 22.9 ± 0.74% to 21.7 ± 1.1% (n=19), exhibiting a non-significant 1.2% decrease in RVD ($P > 0.05$) (Figure 13). When treated with stromatoxin (2 nM), which inhibits $K_v4.2$, the RVD response did not change from 22.9 ± 0.74% to 22.9 ± 2.85% ($P > 0.05$) (Figure 12B and Figure 13). When exposed to astemizole (1 nM), which can inhibit $K_v11.1$, the RVD

response increased from 22.9 ± 0.74% to 24.6 ± 3.38%, exhibiting a non-significant 1.7% increase in RVD ($P > 0.05$) (Figure 13). In the presence of E-4031 (14 nM), which also inhibits $K_v11.1$, the RVD response decreased from 22.9 ± 0.74% to 6.3 ± 1.3%, exhibiting a significant 16.6% decrease in RVD ($P < 0.05$) (Figure 12C and Figure 13). In the presence of paxilline (2 nM), which inhibits $K_{Ca}1.1$, the RVD response decreased from 22.9 ± 0.74% to 5.7 ± 1.8%, exhibiting a significant 17.2% decrease in RVD ($P < 0.05$) (Figure 12D and Figure 13). Finally, when exposed to TRAM-34 (20 nM), which inhibits $K_{Ca}3.1$, the RVD response decreased from 22.9 ± 0.74% to 9.2 ± 2.1%, exhibiting a significant 13.7% decrease in RVD ($P < 0.05$) (Figure 13). Therefore, members of the K_v7 family, in particular $K_v7.1$, as well as $K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$ all play a role in regulating cell volume in times of hyposmotic stress.

3.8 $K_v7.1$ channels affect the RVD response in HBE cells.

Once it was evident that XE991 and 293B had similar effects on the RVD response, it was hypothesized that $K_v7.1$ was the member of the K_v7 family that played a role in cell volume regulation in times of hyposmotic stress in HBE cells. In order to test this hypothesis, siRNA targeting for $K_v7.1$ and $K_v7.5$ was attempted, however this was unsuccessful, as none of the three provided siRNA duplexes decreased the protein expression in HBE cells as compared to controls (Figure 14). Next, pharmacological means were used to test this hypothesis, and cells were initially bathed in isosmotic solution (327.3 ± 0.46 mOsM) and then switched to hyposmotic solutions (180.3 ± 0.46 mOsM) containing XE991 and 293B together (0.75 μM and 5 μM respectively) (Table 2). The RVD response decreased from 22.8 ± 0.7% to 5.2 ± 1.4%, exhibiting a significant 17.6% decrease in RVD ($P < 0.05$). Figure 15A represents the fold change in volume curves for this experiment, while Figure 15B represents the graphical summary of these results. This suggests that $K_v7.1$ is the K_v7 channel member involved in the RVD response, and molecular experiments were then performed to further investigate this conclusion. The pIRES2-EGFP-ΔN- $K_v7.1$ isoform and the pIRES2-EGFP vector control were transfected into HBE cells and after 36-48 hours the RVD response of these cells was examined. Cells were initially bathed in isosmotic solution (327.3 ± 0.46 mOsM) and then switched to hyposmotic solutions (180.3 ± 0.46 mOsM). The RVD response

decreased from 29.3 ± 7.0% in the pIRES2-EGFP vector controls to 3.94 ± 1.3% in the pIRES2-EGFP-ΔN- K_v7.1 transfected cells, exhibiting a significant 25.36% decrease in RVD ($P < 0.05$) (Figure 16). Therefore, K_v7.1 plays an important role in regulating cell volume in times of hyposmotic stress.

3.9 CFBE cells do not elicit an RVD response when exposed to hyposmotic stress.

Once the involvement of K^+ channels was assessed in the RVD response of HBE cells, CFBE cells were then investigated. After switching CFBE cells from an isosmotic solution (327.3 ± 0.46 mOsmol/L) to a more hyposmotic solution (180.3 ± 0.46 mOsmol/L), it was shown that the cells increased in size, but produced a negligible RVD response that measured 3.03 ± 1.09% (Figure 17A). The osmolarity was then lowered (124.2 ± 0.49 mOsM) to produce a more hyposmotic shock in order to achieve an RVD response in these cells, but again the cells produced a negligible RVD response of 0.68 ± 0.54% (Figure 17B). It is evident however, that CFBE cells swell to a much larger extent when they are exposed to this severe hyposmotic shock. It was therefore concluded that CFBE cells, like CF tracheal epithelial cells, do not elicit an RVD response (Vazquez *et al*., 2001) and therefore, the dysfunction in their CFTR channel must have an effect on the cell volume regulatory mechanism of RVD.

3.10 HBE and CFBE cells do not elicit an RVI response when exposed to hyperosmotic solutions.

Experiments were also performed to investigate the RVI response in both HBE and CFBE cells. Initially, cells were placed in an isosmotic solution that measured 327.3 ± 0.46 mOsmol/L. When HBE cells were switched to a more hyperosmotic solution of 482.3 ± 0.41 mOsM, the cells decreased in size quite quickly; however, they never recovered from this decrease and elicited a negligible RVI response of 1.10 ± 0.7% (Figure 18A). The cells were then exposed to a more severe hyperosmotic shock of 528.5 ± 0.43 mOsM and again elicited a negligible RVI response of 0.67 ± 0.67% (Figure 18B). When CFBE cells were exposed to hyperosmotic solutions of 482.3 ± 0.41 mOsM, they did not produce a measurable RVI response (0.07 ± 0.05%) (Figure 18C). When the osmolarity was changed to create a larger hyperosmotic shock (528.5 ± 0.43 mOsM), the CFBE cells underwent a negligible RVI response measuring 2.25 ± 1.11% (Figure 18D).

Therefore HBE and CFBE cells do not undergo an RVI response when exposed to hyperosmotic stress.

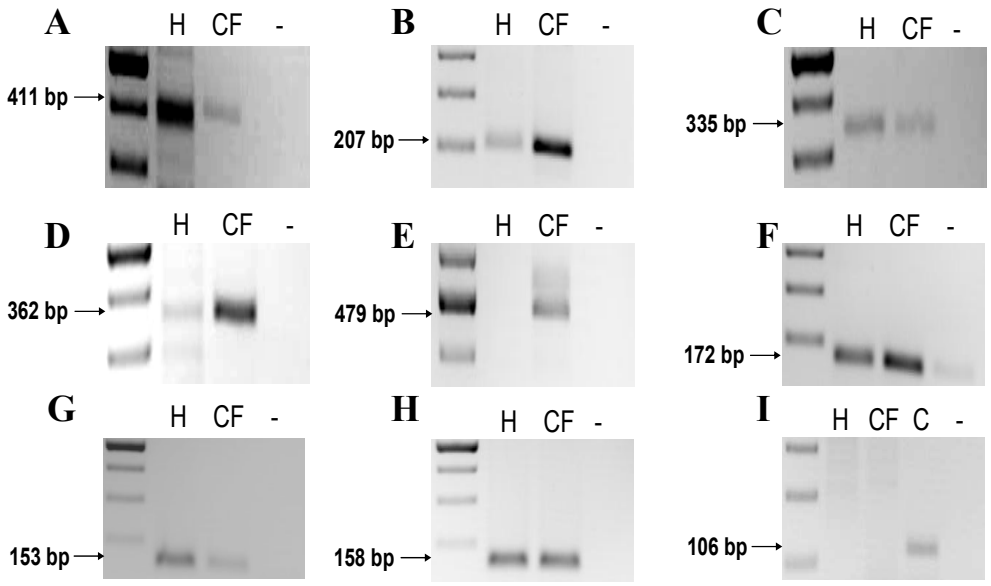


Figure 4. Multiple K^+ channel mRNA transcripts are expressed in HBE and CFBE cells. RT-PCR was performed and detected transcripts in HBE (H) and CFBE (CF) cells for $K_v7.1$ (A), $K_v7.5$ (B), $K_v4.1$ (C), $K_v4.2$ (D), $K_v11.1$ (F), $K_{Ca}1.1$ (G), and $K_{Ca}3.1$ (H). Transcripts for $K_v10.1$ were detected in CFBE cells, but not in HBE cells (E). Transcripts for $K_v4.3$ were not detected in HBE or CFBE cells; however Calu-3 (C) cells did express $K_v4.3$ mRNA (I). No transcripts were detected for the negative RT samples (-). HPRT was used as a positive control in order to test the integrity of the cDNA (not shown). The DNA ladder is shown on the left side of each panel and the expected size for each amplicon is shown by the arrows. The primer sequences and annealing temperatures are listed in Table 3.

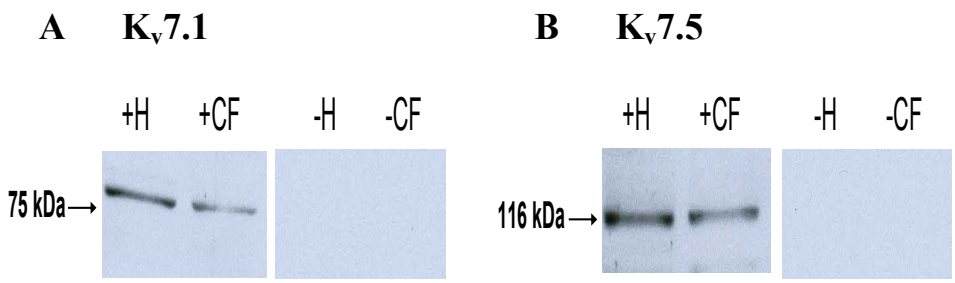


Figure 5. K_v7.1 and K_v7.5 protein are expressed in HBE and CFBE cells. Western blots were performed on total cell lysates and protein was detected for (A) K_v7.1 at 75 kDa and (B) K_v7.5 at 116 kDa in both HBE (+H) and CFBE (+CF) cells. Negative controls (–) were performed by omitting the primary antibody and no protein was detected in these immuno-blots for either cell line. Details for the primary and secondary antibodies are described in Table 4.

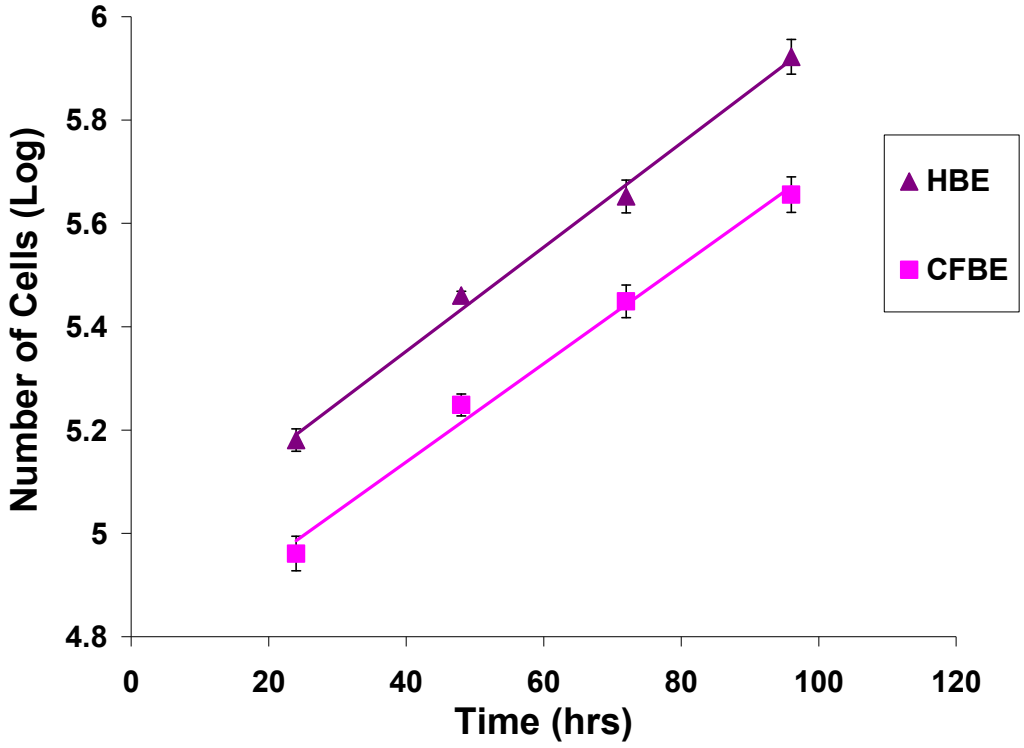
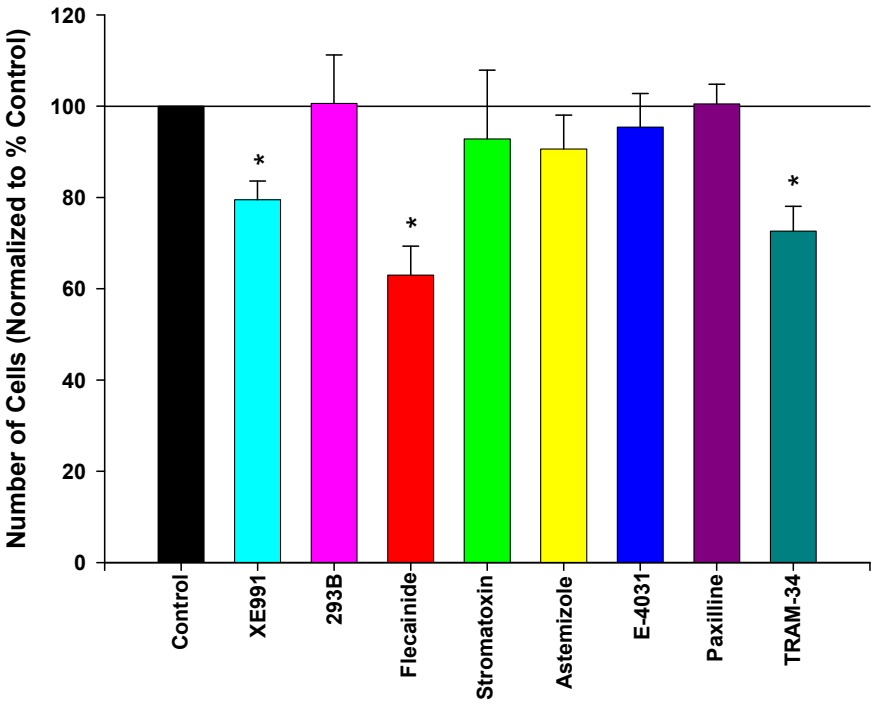


Figure 6. Cell doubling time for HBE and CFBE cells. The amount of time it took for cells to double in number was investigated in order to begin proliferation studies. Cells were plated at t=0 and counted every 24 hr for 96 hr, and the log value of the cell number was plotted. The linear regression equation was determined (HBE: $y = 0.0101x + 4.9495$; CFBE: $y = 0.0095x + 4.7574$) and the doubling time was calculated by dividing 0.3 by the slope for each cell line (NIH Chemical Genomic Center, 2009). HBE cells had a doubling time of 29.7 hr and CFBE cells had a doubling time of 31.6 hr.

A HBE


Number of Cells (Normalized to % Control)
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100
80
60
40
20
0
*
*
*
Control
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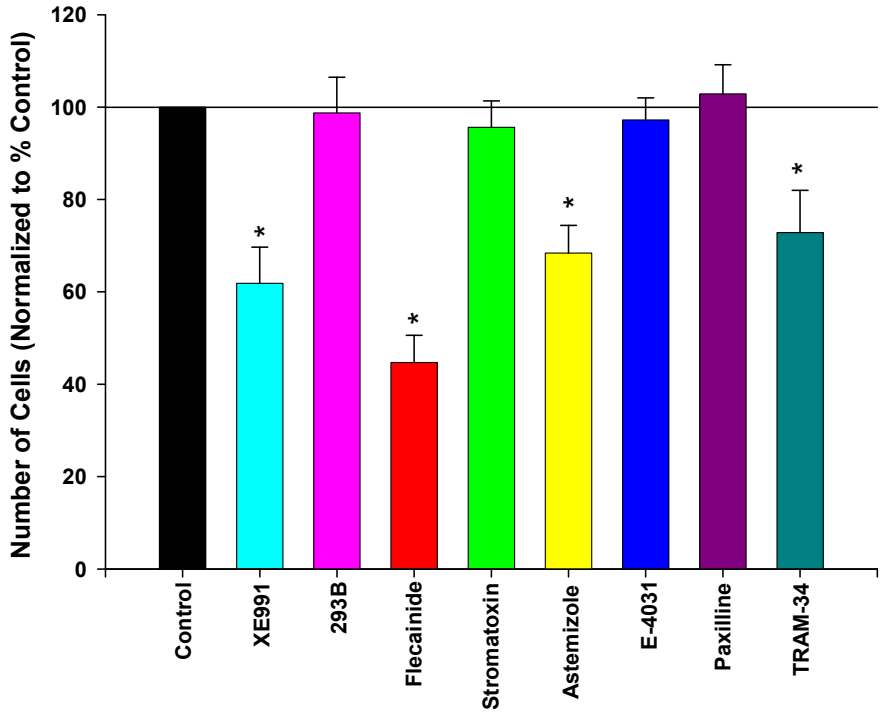
293B
Flecainide
Stromatoxin
Astemizole
E-4031
Paxilline
TRAM-34

B CFBE


Number of Cells (Normalized to % Control)
120
100
80
60
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0
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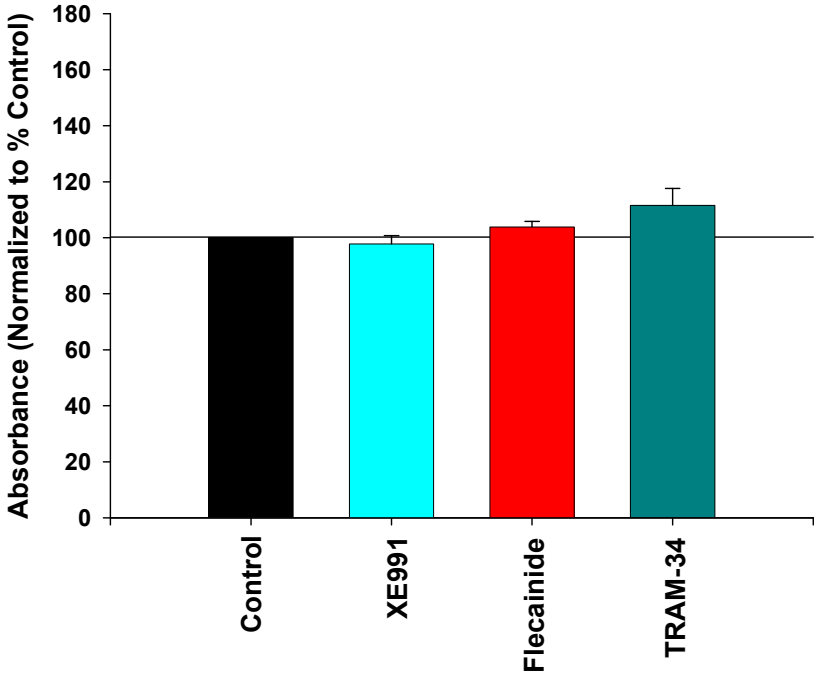
Control
XE991
293B
Flecainide
Stromatoxin
Astemizole
E-4031
Paxilline
TRAM-34

Figure 7. Contribution of K^+ channels to cell proliferation in HBE and CFBE cells. HBE (A) and CFBE (B) cell number was measured using a Coulter Counter to investigate changes in cell proliferation in the presence of specific and non-specific K^+ channel inhibitors for 48 hr. XE991 (100 μM), flecainide (100 μM), and TRAM-34 (10 μM) inhibited cell proliferation in both HBE and CFBE cells, whereas astemizole (3 μM) inhibited cell proliferation in CFBE cells only. Cell number was normalized to control data ± standard error of the mean (SEM). Each experiment was run in triplicate on 3 different occasions (n=3). * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test.

A HBE


Absorbance (Normalized to % Control)
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100
80
60
40
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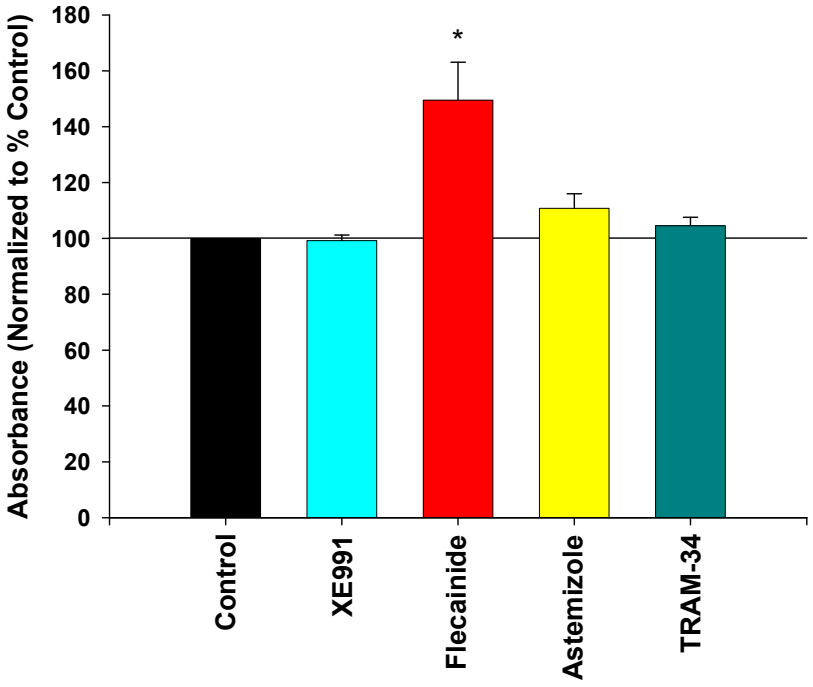
Control
XE991
Flecainide
TRAM-34

B CFBE


Absorbance (Normalized to % Control)
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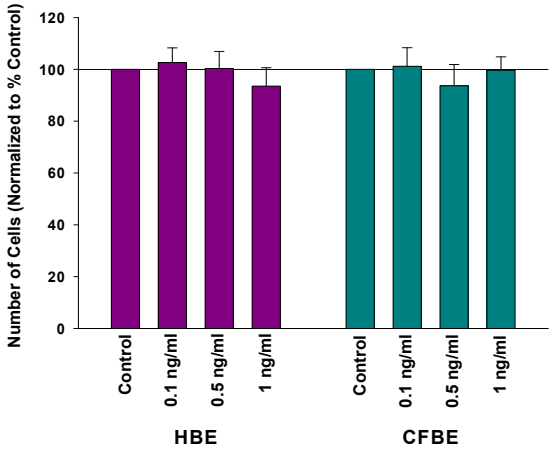
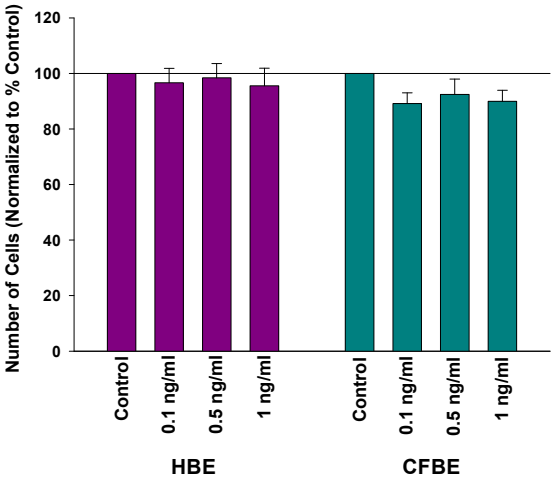
20
0
*
Control
XE991
Flecainide
Astemizole
TRAM-34

Figure 8. Cell viability following treatment with K^+ channel inhibitors. Inhibitors that caused a significant decrease in HBE and CFBE cell number were assessed with an LDH assay to confirm they were not cytotoxic. The same inhibitors and concentrations were used in the cell proliferation studies from Figure 7. In (A) HBE cells none of the inhibitors caused cell death, however in (B) CFBE cells 100 μM flecainide was cytotoxic. LDH absorbance was normalized to control data ± SEM. Each experiment was run in triplicate on three different occasions (n=3). * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test.

A EGF



B IGF-1



C E2

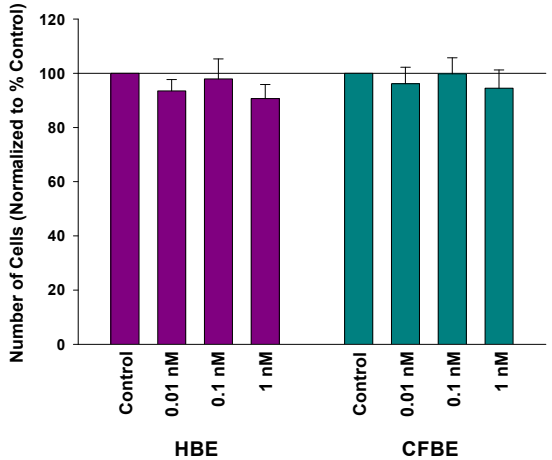


Figure 9. The mitogens EGF, IGF-1 and E2 have no effect on cell proliferation in HBE and CFBE cells. HBE (left) and CFBE (right) cell proliferation was measured using a Coulter Counter in the presence of the mitogens (A) EGF, (B) IGF-1 and (C) E2 for 48 hr. There was no evidence of mitogenic-stimulated cell proliferation in either cells line for any of these potential mitogens, for various concentrations. Concentration responses were performed for all the experiments and cell number was normalized to control data ± SEM. Each experiment was run in triplicate on three different occasions (n=3).

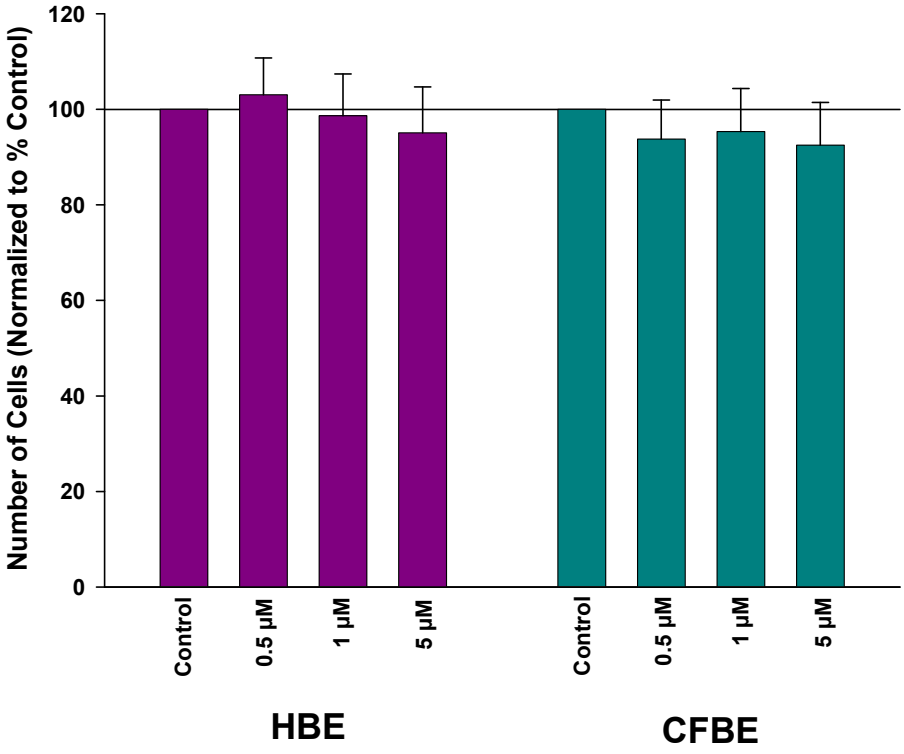
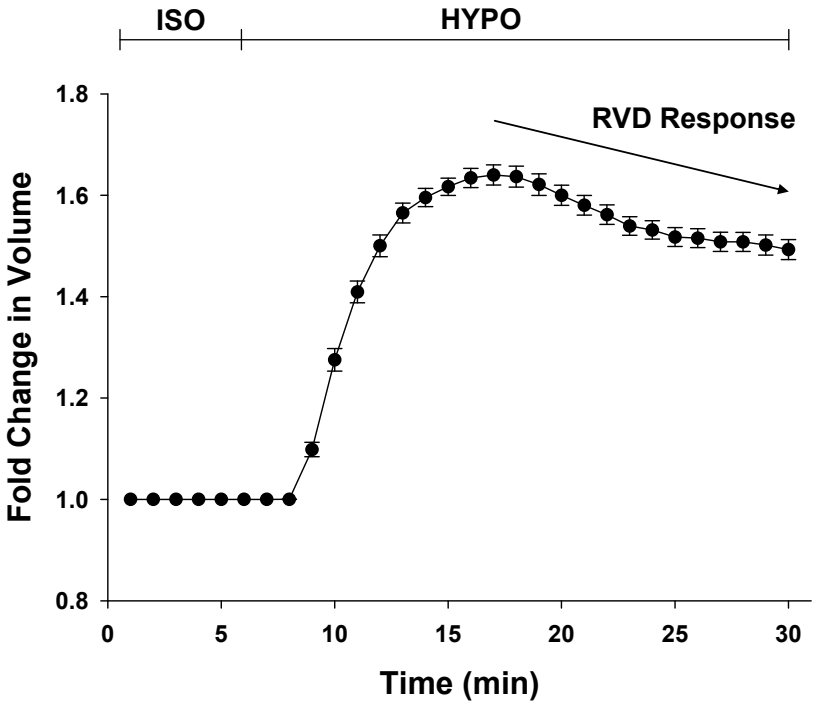
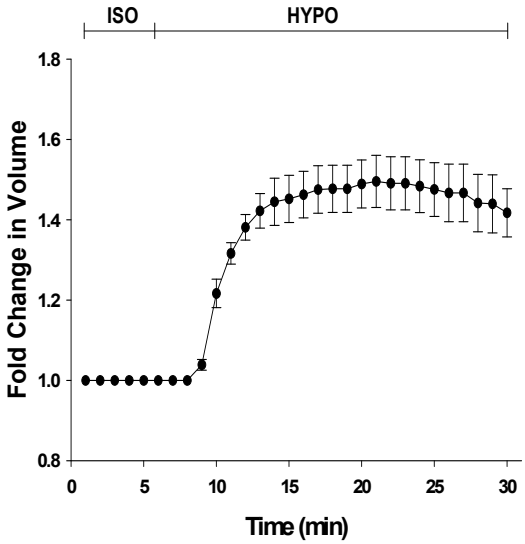


Figure 10. The potential K_v7 activator flupirtine maleate does not affect cell proliferation in HBE and CFBE cells. HBE (left) and CFBE (right) cell proliferation was measured using a Coulter Counter in the presence of the suggested K_v7 activator flupirtine maleate for 48 hr. There was no difference in cell number in either cell line, for various concentrations of flupirtine maleate. Concentration responses were performed for all the experiments and cell number was normalized to control data ± SEM. Each experiment was run in triplicate on three different occasions (n=3).

A **180 mOsM**



B **207 mOsM**



C **260 mOsM**

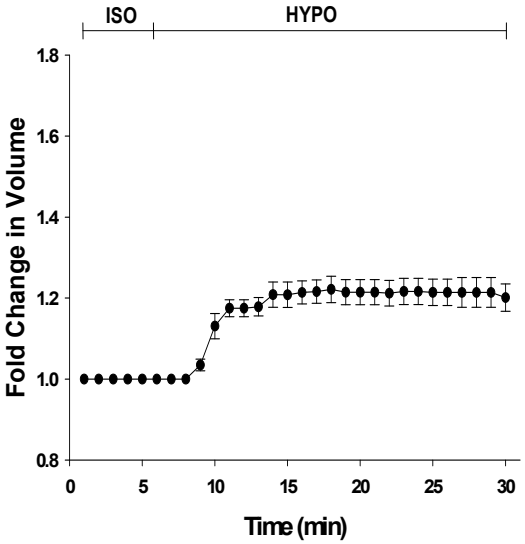


Figure 11. HBE cells swell in response to hyposmotic shock and then undergo varying degrees of RVD depending on the osmolarity. (A) When switched from an isosmotic solution (327 mOsM) to a hyposmotic solution (180 mOsM), HBE cells increased in volume and then underwent an RVD response of 22.9 ± 0.74% (n=131). (B) When switched from an isosmotic solution (327 mOsM) to a less hyposmotic solution (207 mOsM), HBE cells increased in volume and underwent an RVD response, but to a much smaller degree than above (4.72 ± 0.95%; n=8). (C) When switched from an isosmotic solution (327 mOsM) to an even lower hyposmotic solution (260 mOsM), HBE cells increase in volume (to a lesser extent than with lower osmolarities) and produced an RVD response which was even smaller than in (B) (3.31 ± 1.65%; n=8). Cell volume was normalized to that at t = 0 to represent the fold change in volume means ± SEM. ISO=isosmotic; HYPO=hyposmotic; RVD=regulatory volume decrease.

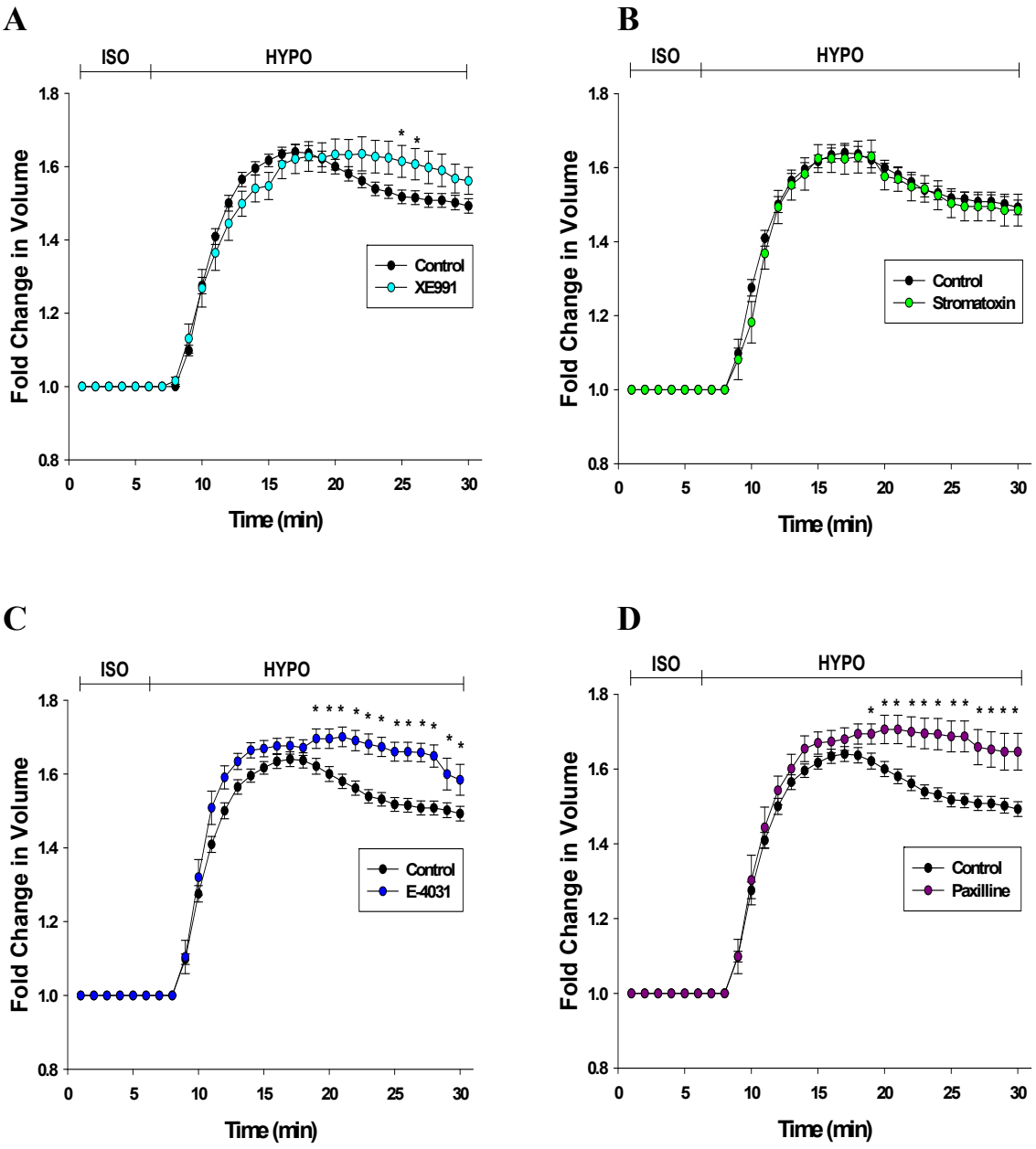


Figure 12. Contribution of various K^+ channels to the RVD response in HBE cells. The RVD response was abolished when the hyposmotic solution was presented with the selective and non-selective inhibitors (A) XE991 (0.75µM; n=17), (C) E-4031 (14nM; n=10) and (D) paxilline (2nM; n=12). The RVD response was not significantly different when the hyposmotic solution was presented with the selective inhibitors (B) stromatoxin (2nM; n=10). Cell volume was normalized to that at t = 0 to represent the fold change in volume means ± SEM. * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test. ISO=isosmotic; HYPO=hyposmotic; RVD=regulatory volume decrease.

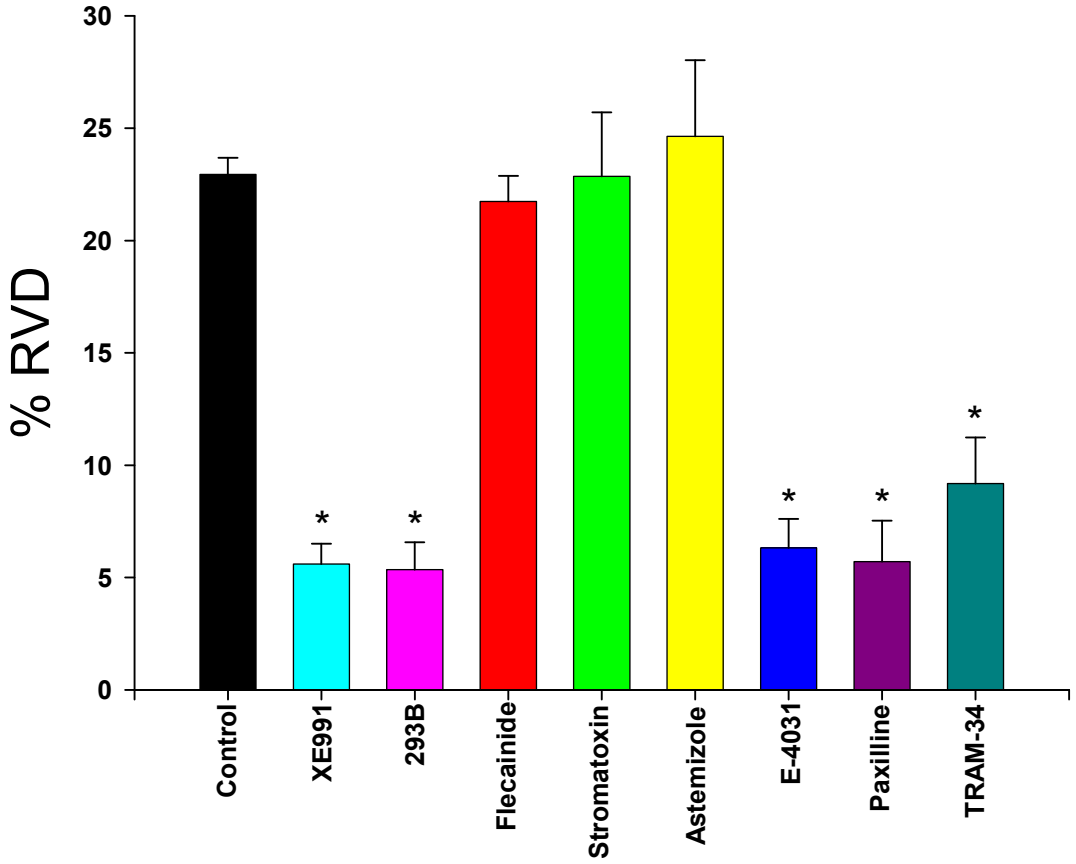


Figure 13. K_v7.1-7.5, K_v11.1, K_{Ca}1.1 and K_{Ca}3.1 play a role in the RVD response in HBE cells. % RVD was measured in the presence of specific and non-specific K^+ channel inhibitors. XE991 (0.75 μM; n=17), 293B (5 μM; n=14), E-4031 (14 nM; n=10), paxilline (2 nM; n=12), and TRAM-34 (20 nM, n=10) significantly inhibit the RVD response. The RVD response was not significantly different when the hyposmotic solution was treated with flecainide (50 μM; n=9), stromatoxin (2 nM; n=10), or astemizole (1 nM; n=16). Cell volume was normalized to that at t = 0 to represent the % RVD ± SEM. * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test. RVD=regulatory volume decrease.

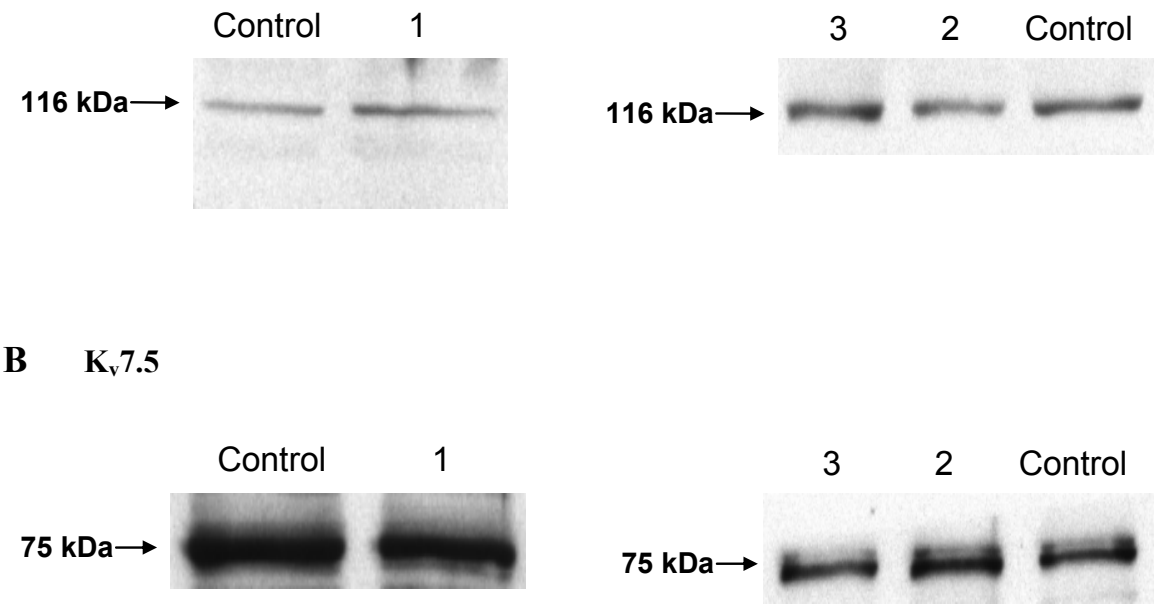


Figure 14. K_v7.1 and K_v7.5 siRNA was unsuccessful at producing significant suppression of respective K^+ channel protein in HBE cells. Western blots were performed to analyze the siRNA knockdown efficiency of (A) K_v7.1 and (B) K_v7.5 channel protein. Three siRNA duplexes (1-3) were provided and tested against control samples that contained siTrans reagent alone. Neither of the three provided duplex showed a decrease in protein compared to control.

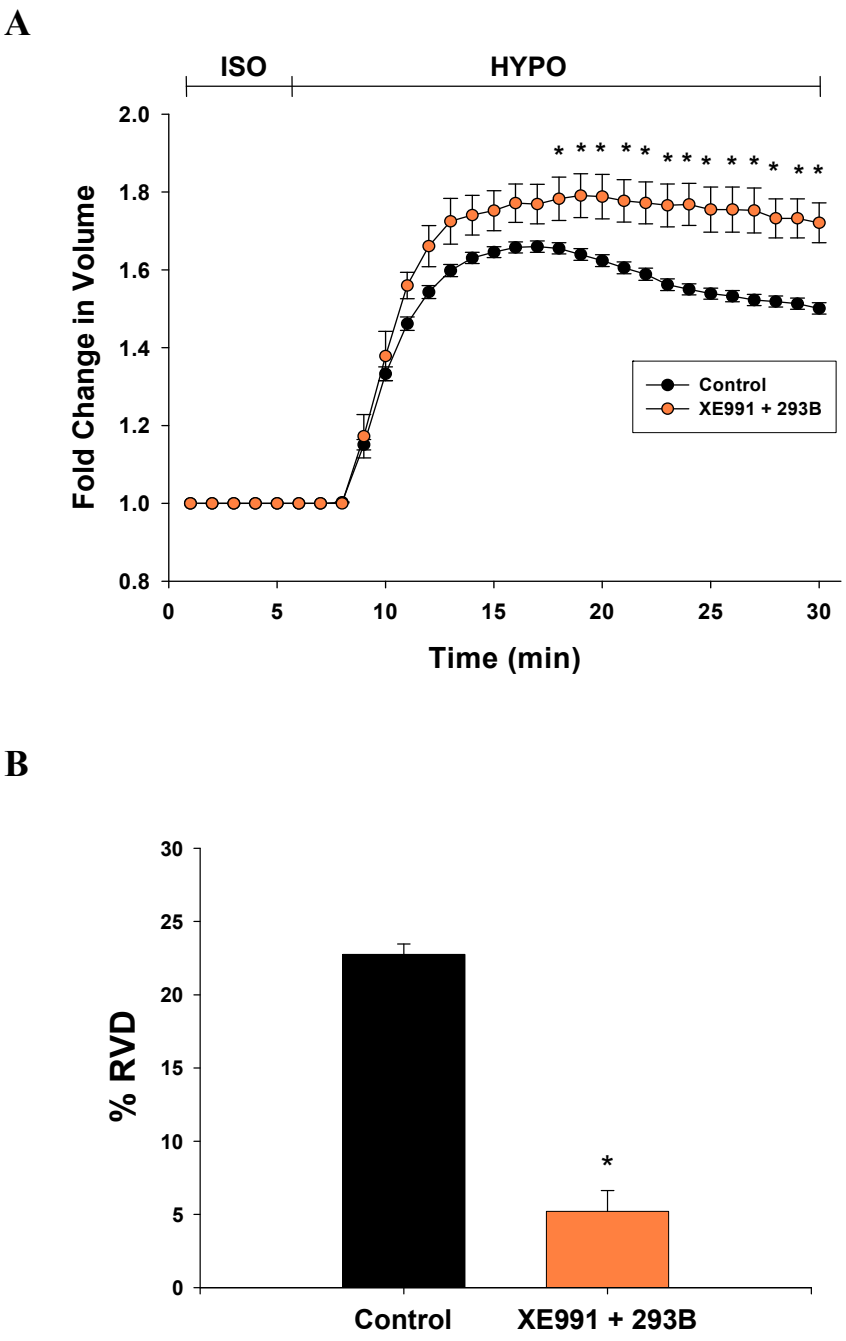


Figure 15. $K_v7.1$ plays a role in the RVD response in HBE cells through pharmacological means. RVD was measured in the presence of the non-specific and specific K_v7 channel inhibitors XE991 + 293B (0.75 µM and 5 µM respectively, n=9). % RVD was significantly lower in HBE cells treated with these inhibitors. Cell volume was normalized to that at t = 0 to represent the fold change in volume means ± SEM (A) and the % RVD ± SEM (B). * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test. ISO=isosmotic; HYPO=hyposmotic; RVD=regulatory volume decrease.

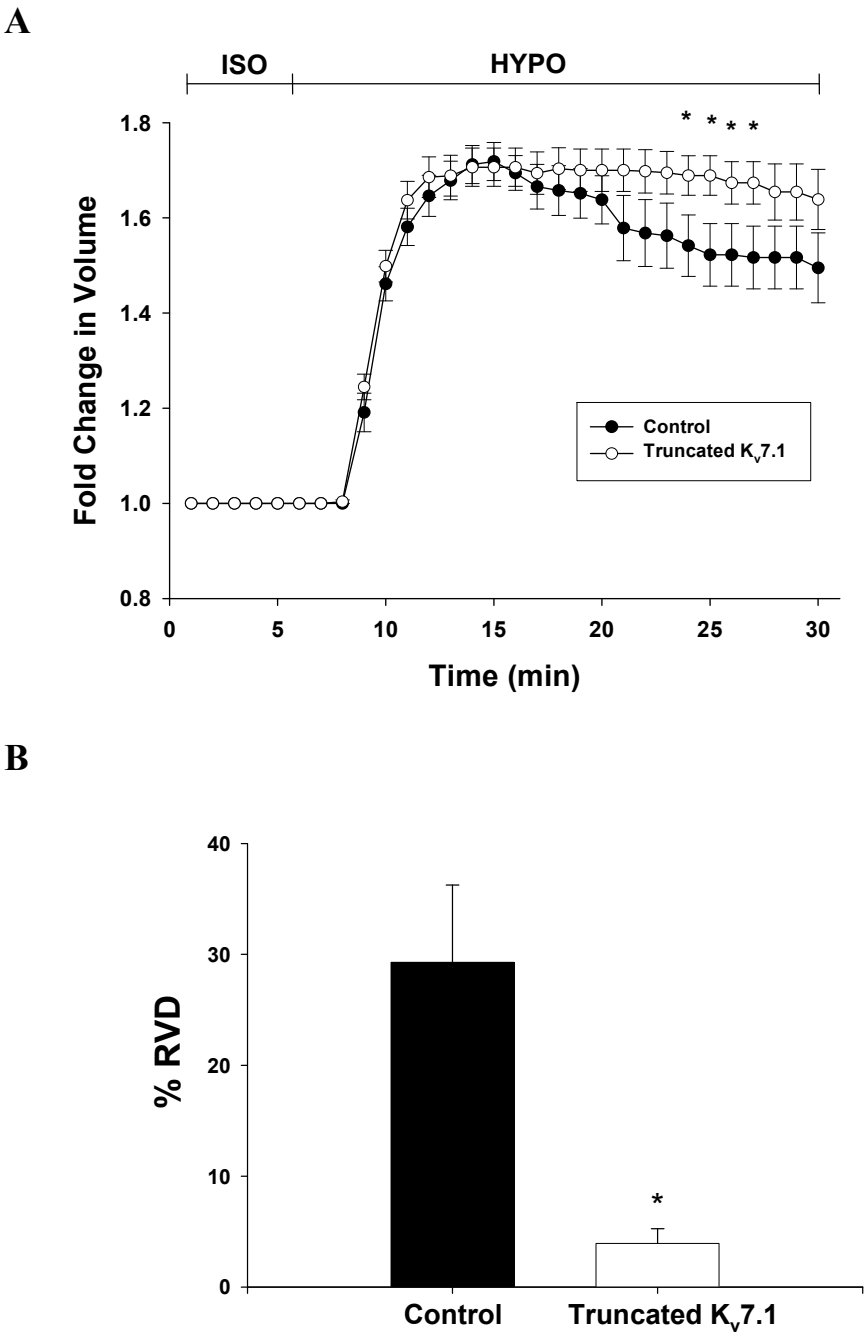


Figure 16. K_v7.1 plays a role in the RVD response in HBE cells through molecular means. RVD was measured by the suppression of K_v7.1 channels using the truncated pIRES2-EGFP-ΔN-K_v7.1 mutation (n=8). % RVD was significantly lower in HBE cells transfected with this dominant-negative mutation. Cell volume was normalized to that at t = 0 to represent the fold change in volume means ± SEM (A) and the % RVD ± SEM (B). * represents $P < 0.05$ when compared to controls, as performed by an ANOVA, followed by a Student's t-test. ISO=isosmotic; HYPO=hyposmotic; RVD=regulatory volume decrease.

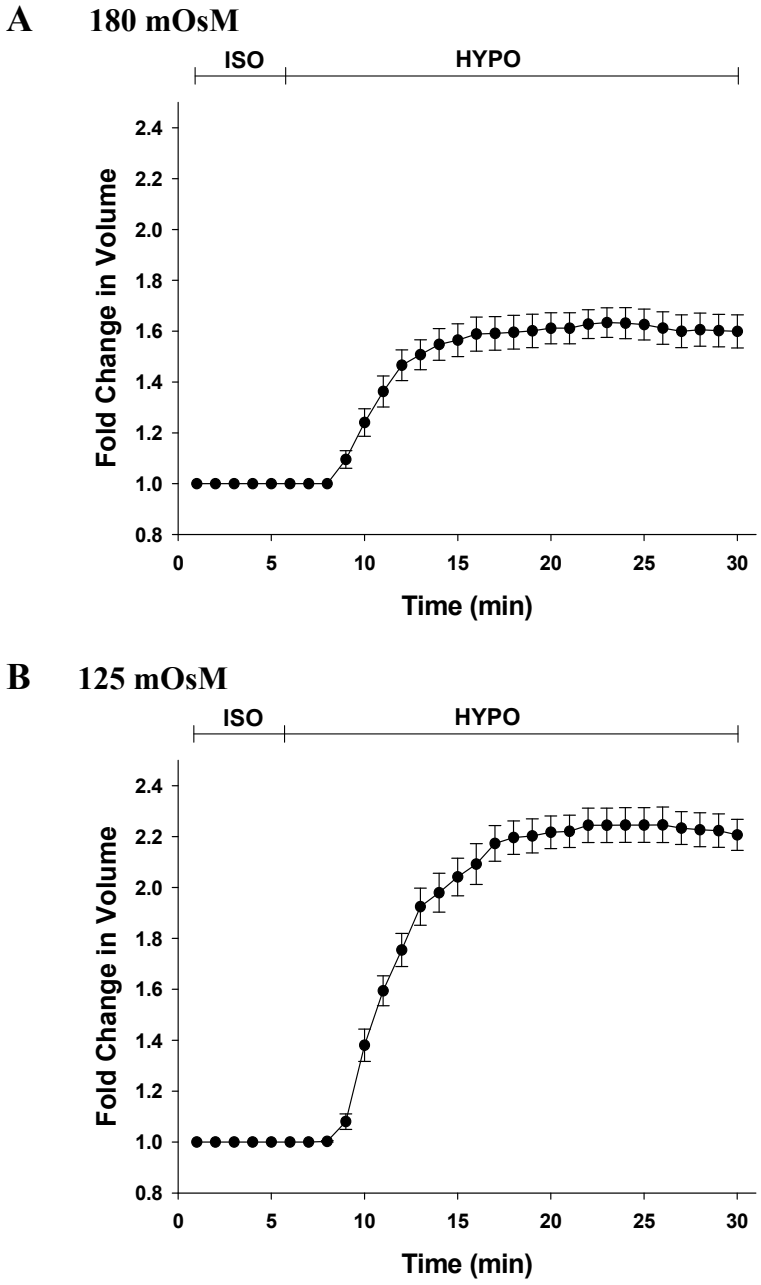


Figure 17. CFBE cells do not undergo a significant RVD response when exposed to varying degrees of hyposmotic stress. (A) When switched from an isosmotic solution (327 mOsM) to a hyposmotic solution (180 mOsM), CFBE cells increased in volume, and produced a negligible RVD response of 3.03 ± 1.09% (n=11). (B) When switched from an isosmotic solution (327 mOsM) to a more hyposmotic solution (125 mOsM), in order to attempt to elicit an RVD response, CFBE cells showed to increase much more in volume, however they produced a smaller RVD response of 0.68 ± 0.54% (n=10). Cell volume was normalized to that at t = 0 to represent fold change in volume means ± SEM. ISO=isosmotic; HYPO=hyposmotic; RVD=regulatory volume decrease.

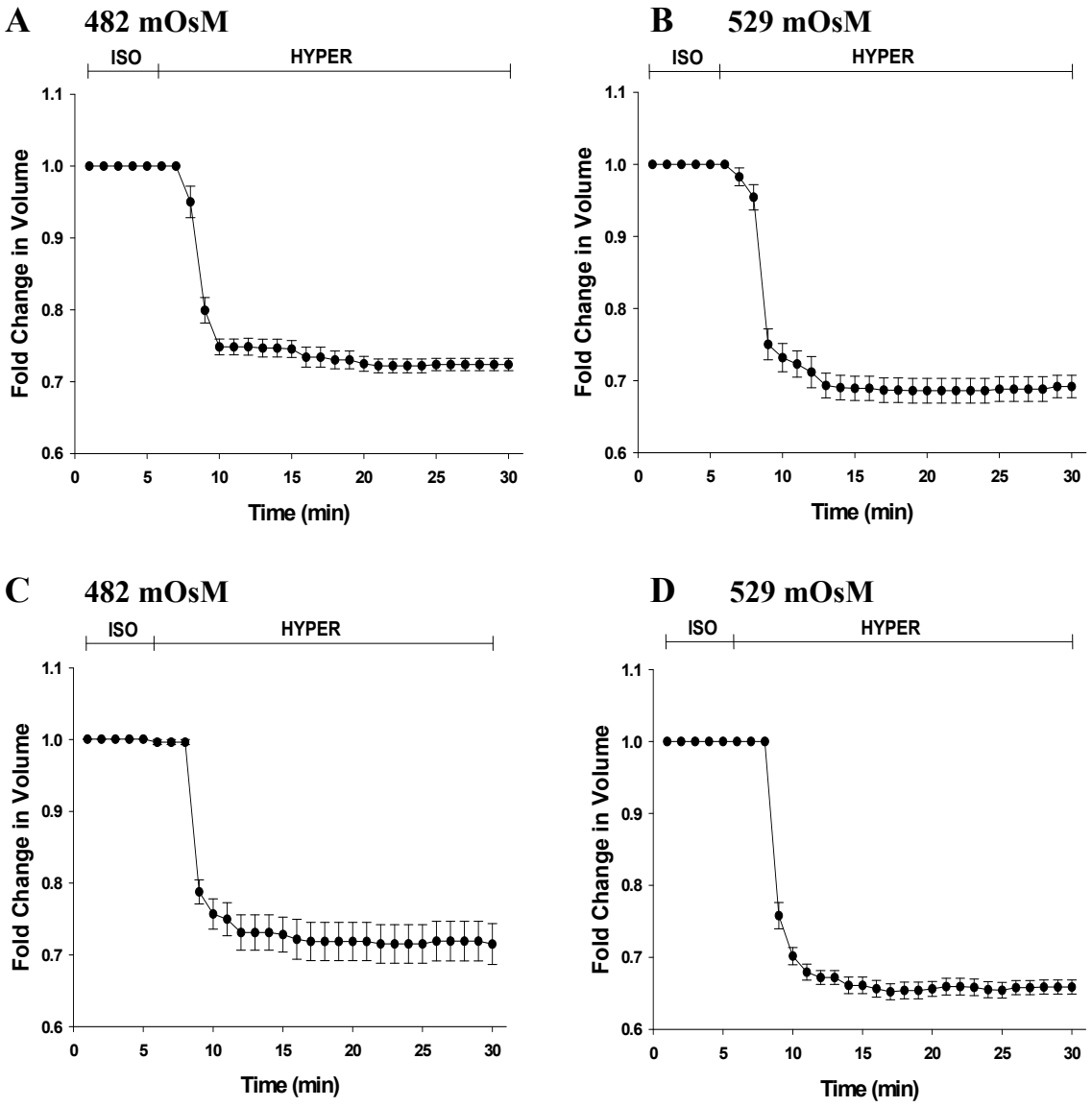


Figure 18. HBE and CFBE cells do not undergo an RVI response when exposed to varying degrees of hyperosmotic stress. (A) When switched from an isosmotic solution (327 mOsM) to a hyperosmotic solution (482 mOsM), HBE cells decreased in volume and elicited a negligible RVI response of 1.10 ± 0.7% (n=12). (B) When switched from an isosmotic solution (327 mOsM) to a more hypersmotic solution (529 mOsM), HBE cells decreased in volume and elicited an even more negligible RVI response of 0.67 ± 0.67% (n=15). (C) When switched from an isosmotic solution (327 mOsM) to a hyperosmotic solution (482 mOsM), CFBE cells decreased in volume and produced a negligible RVI response of 0.07 ± 0.05 (n=9). (D) When switched from an isosmotic solution (327 mOsM) to a more hyperosmotic solution (529 mOsM), CFBE cells decreased in volume and produced a larger, but still negligible RVI response of 2.25 ± 1.11% (n=12). Cell volume was normalized to that at t = 0 to represent fold change in volume means ± SEM. ISO=isosmotic; HYPER=hyperosmotic; RVI=regulatory volume increase.

CHAPTER 4: DISCUSSION

K^+ channels play an important role in cell proliferation and cell volume regulation in many different types of epithelial cells (Trinh *et al*., 2007; vanTol *et al*., 2007; Roy *et al*., 2008; Trinh *et al*., 2008; Harron *et al*., 2009). Specifically, members of the K_v and K_{Ca} channel family, including $K_v7.1$, $K_v7.5$, $K_v4.1$, $K_v4.2$, $K_v4.3$, $K_v10.1$, $K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$ have been implicated in these physiological processes in a variety of non-epithelial and epithelial cell types (Vazquez *et al*., 2001; Fernandez-Fernandez *et al*., 2002; Jensen *et al*., 2005; Trinh *et al*., 2007; vanTol *et al*., 2007; Roy *et al*., 2008; Trinh *et al*., 2008; Harron *et al*., 2009; Jang *et al*., 2009; Kim *et al*., 2010). Therefore, it appeared reasonable to assume that members of these particular K^+ channel families were also important in cell proliferation and cell volume regulation in human bronchial epithelial cells. However, the precise molecular nature of the K^+ channels involved in these processes had not been investigated in the two model cell lines chosen for this study.

I chose to carry out my study with the widely used human 16HBE14o- (HBE) and CFBE41o- (CFBE) model cell lines. The HBE cell line is derived from normal bronchial epithelial cells, while the CFBE cell line represents CF bronchial epithelial cells containing the ΔF508 CFTR mutation. Although both HBE and CFBE cells have many characteristics of native human bronchial epithelial cells (Ehrhardt *et al*., 2002; Ehrhardt *et al*., 2005), they are not primary cultures and therefore do not entirely represent true bronchial epithelial cells. These cell lines are however useful because they are easily maintained and allow for high reproducibility between experiments under appropriate culture conditions. HBE cells develop tight junctions when correctly cultured, are able to become polarized and express functional CFTR protein, which is important when studying normal airway epithelial physiology (Ehrhardt *et al*., 2002). CFBE cells are homozygous for the ΔF508 CF-causing CFTR mutation and also develop tight junctions and form cell layers that are electrically tight (Ehrhardt *et al*., 2005). These characteristics are also important when studying CF airway epithelial physiology. Both these cell lines represent good models for my study, not only because it required a large number of reproducible cells, but also because as mentioned above they share many similar

characteristics to their native counterparts. Although it would have been ideal to have had access to primary tissue from the human bronchial epithelium, these preparations were not available for my project.

4.1 Expression of K^+ channels in HBE and CFBE cells.

Although the most studied airway epithelial ion channels are currently CFTR and ENaC due to the fact that they are the key ion channels involved in airway transepithelial transport, K^+ channels are becoming increasingly studied, as they play an important role in many airway epithelial physiological processes (Bardou *et al*, 2009). Since $K_v7.1$, $K_v7.5$, $K_v4.1$, $K_v4.2$, $K_v4.3$, $K_v10.1$, $K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$ play a functional role in cell proliferation and cell volume regulation in many different cell types; I began this study by confirming their mRNA expression in HBE and CFBE cells. Using PCR, I detected the mRNA expression of $K_v7.1$, $K_v7.5$, $K_v4.1$, $K_v4.2$, $K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$ in HBE cells, while in CFBE cells all of these K^+ channels were also expressed, along with $K_v10.1$ (Figure 4).

I was unable to detect mRNA expression for $K_v4.3$ in either HBE or CFBE cells, however it was detected in another airway epithelial cell line Calu-3, therefore there was not a problem with the chosen primers. The integrity of the cDNA was also tested using the housekeeping gene HPRT and produced distinct amplicons at the correct bp size, signifying good quality cDNA. This channel may not be expressed in these cell lines because the primary transepithelial mechanism in Calu-3 cells is Cl^- secretion, whereas the dominant transepithelial ion movement in HBE and CFBE cells is more likely Na^+ absorption (Harron *et al*., 2009). One possibility to explain the lack of $K_v4.3$ mRNA expression in HBE and CFBE cells may be that this channel is more important in processes involved in anion secretion.

I was also only able to detect mRNA expression for $K_v10.1$ in CFBE cells, and not in HBE cells. Again the reason for this is not entirely clear; however, it has been previously reported that this channel is not expressed in some normal tissue but is expressed in their cancerous tissue parallels (Pardo *et al*., 1999; Hemmerlein *et al*., 2006). Perhaps this channel plays a larger role in pathophysiological processes rather than in normal physiology, or it may merely reflect a difference between HBE and CFBE cells.

Also, the protein expression of this channel was not investigated, so perhaps there is no protein and solely message for $K_v10.1$ in CFBE cells; however further functional studies must be performed to investigate the role of $K_v10.1$ in CF airway epithelial cells.

I next wanted to investigate whether the mRNA I detected for my K^+ channels of interest was translated into protein. In this, I was restricted to antibodies that were commercially available; furthermore, many of my chosen K^+ channels have showed to have non-specific antibodies (Cowley lab, unpublished data), therefore I chose to focus on $K_v7.1$ and $K_v7.5$. Through immunoblotting, both $K_v7.1$ and $K_v7.5$ protein were detected in HBE and CFBE cells (Figure 5). In summary, HBE and CFBE cells both express $K_v7.1$, $K_v7.5$, $K_v4.1$, $K_v4.2$, $K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$ mRNA, as well as protein for $K_v7.1$ and $K_v7.5$. Furthermore, unlike HBE cells, CFBE cells express mRNA for $K_v10.1$. Since I could not investigate the protein expression for the remaining K^+ channels, their expression in these cell lines cannot be confirmed; however, the studies described below do support their expression as functional channels in HBE and CFBE cells.

4.2 The activity of K^+ channels in HBE and CFBE basal cell proliferation.

As previously mentioned (see Section 1.6), the relationship between K^+ channel activity and cell proliferation is still not completely understood. However it has been concluded that K^+ channels play a role in mediating the cell cycle since they are required to hyperpolarize the membrane potential (Wonderlin and Strobl, 1996). In order for epithelial cells to regenerate after they are wounded, a major step that must occur is cell proliferation and in order for cell proliferation to take place, the cell must adequately and efficiently progress through the cell cycle. Since it has been reported that K^+ channels are involved in cell-cycle progression, their role in human bronchial epithelial cells is potentially of interest. Reports have suggested that K^+ channels primarily play a role in the progression of the cell through the G_1 phase of the cell cycle (Wonderlin and Strobl, 1996; Ouadid-Ahidouch *et al*., 2001; Roy *et al*., 2008); therefore, I first attempted to synchronize HBE and CFBE cells in this particular phase by measuring the doubling time of each cell line. Other experiments have used means such as serum-starvation and flow cytometry analysis, which measures the amount of DNA in the cell and can distinguish

between cell cycle phase, to synchronize cells in G_1 (Gray *et al*., 1986; Roura-Ferrer *et al*., 2008; Roy *et al*., 2008). However, because I did not have access to flow cytometry instruments and the cells began to die when left in serum-free culture media for an extended period of time, it was proposed that another means to partially synchronize these cells in G_1 was to calculate their doubling time. This was performed by performing cell counting assays and treating the cells with pharmacological inhibitors before they doubled. Both cell lines doubled by 32 hours (Figure 6); therefore, I treated them both with K^+ channel inhibitors 24 hours after they were plated. Although this technique may not be as specific as others (as mentioned above), for my purposes it was efficient and yielded reproducible results.

To determine whether the K^+ channels of interest to my study contributed a functional role to basal cell proliferation in HBE and CFBE cells, cell counting experiments using a Coulter Counter were performed as a means to assess proliferation. Pharmacological inhibitors of these specific K^+ channels were applied to the cells in order to investigate the extent that these channels are involved in this physiological process. Differences were apparent between cell lines with regard to which specific K^+ channels were involved. In HBE cells, K_v7.2-7.5, K_v4.1 and K_{Ca}3.1 channels played a role in basal cell proliferation, while K_v7.2-7.5, K_v10.1, and K_{Ca}3.1 channels were involved in this process in CFBE cells.

4.2.1 The role of K_v7 channels in HBE and CFBE cell proliferation.

In both HBE and CFBE cells, members of the K_v7 channel family other than K_v7.1 appear to be involved in basal cell proliferation, since XE991 (which inhibits all members of the K_v7 family) decreased cell proliferation, and chromonal 293B (which specifically inhibits K_v7.1) did not (Figure 7). This finding is in disagreement with previous literature from the normal and CF bronchial epithelial cell lines NuLi and CuFi, in which K_v7.1 inhibition did affect cell proliferation, measured by [^{3}H]thymidine incorporation. This technique measures DNA synthesis and is another means of assessing cell proliferation and was decreased in the presence of the K_v7.1 inhibitor clofilium (Trinh *et al*., 2008). However, this experiment was performed in combination with two other K^+ inhibitors; therefore, the decrease in proliferation that was seen may not be

specifically due to K_v7.1, or it may reflect differences between the cell lines used in each study. Additionally, clofilium is not as selective as 293B as a K_v7.1 inhibitor (Manikkham *et al*., 2002), so despite their conclusions, K_v7.1 may not actually be involved in cell proliferation in the Trinh study. Since XE991 does have an effect on cell proliferation, suggesting a role for one or all K_v7.2-7.5 channels in this process, further experiments must be performed to delineate specifically which K_v7 channel member is at work. Although K_v7.2-7.5 channels are more involved in the central nervous system (CNS) and auditory organs (Robbins, 2001), there is evidence that K_v7.5 plays a role in the proliferation of skeletal muscle cells (Roura-Ferrer *et al*., 2008). One way to investigate whether K_v7.5 is also important in human bronchial epithelial cells would be to target the channel with siRNA and examine the effects on cell proliferation. However, since K_v7.5 can co-assemble with K_v7.3 (Lerche *et al*., 2000) and reportedly K_v7.4 (Bal *et al*., 2008), simply performing siRNA targeting could be difficult. K_v7.5 has also been reported to play a role on its own in basal transepithelial anion secretion in Calu-3 cells (Moser *et al*., 2008), therefore siRNA targeting is plausible. However, as will be mentioned below siRNA targeting for both K_v7.1 and K_v7.5 was attempted for several months during this project but were unsuccessful. Although K_v7.5 has the ability to function on its own, there is also the possibility that it may form a hetermeric complex with K_v7.3 or K_v7.4. However, although I did not detect mRNA expression for K_v7.3 in HBE or CFBE cells, they do express K_v7.2 and K_v7.4 channels (Moser *et al*., 2008), therefore in order to investigate the role of K_v7.5 in cell proliferation it must be noted that this channel may be forming a heteromeric complex with K_v7.4. Future experiments could include co-immunoprecipitation in order to pull down this heteromeric complex and investigate it's molecular properties. This study represents the first time K_v7.5 channels have been examined in human bronchial epithelial cell proliferation, and further investigation may delineate a role for this channel in this process.

4.2.2 The role of K_v4, K_v10 and K_v11 channels in HBE and CFBE cell proliferation.

In HBE cells, K_v4.1 appears to play a role in cell proliferation since flecainide (which inhibits K_v4.1-4.3) decreased cell number and stromatoxin (which selectively inhibits K_v4.2) did not (Figure 7). Since K_v4.3 mRNA was not detected in either cell line,

flecainide is presumably acting on $K_v4.1$ to inhibit cell proliferation in HBE cells. These results are in agreement with reports from studies in both cancerous gastric cells and mammary epithelial cells, which implicate $K_v4.1$ in cell proliferation using siRNA targeting (Jang *et al*., 2009; Kim *et al*., 2010). A decrease in cell number was also shown in CFBE cells that were treated with flecainide but not stromatoxin; however when cytotoxicity assays were performed I discovered that this effect was due to inhibitor-induced cell death (Figure 7 and 8). Therefore, perhaps $K_v4.1$ is playing a role in cell proliferation in CFBE cells, but maybe CFBE cells are not as robust as HBE cells in terms of their membrane integrity and overall survival due to their defective CFTR protein. This may cause the presence of flecainide to kill the cells and mask any effect $K_v4.1$ channels may have on cell proliferation. Lower dosages of flecainide were used but they did not induce a decrease in cell number, therefore in order to investigate the role of $K_v4.1$ in CFBE cells it may be necessary to use different pharmacological inhibitors or to move away from pharmacological means all together, which may be causing cell death, to more molecular means such as siRNA targeting. Another notable K_v4 channel antagonist is bupivacaine, however this inhibitor also acts on members of the 2-pore domain family of K^+ channels as well as certain Na^+ channels (Harron *et al*., 2008), therefore I opted for flecainide due to its relative selectivity. In summary, a role for $K_v4.1$ is apparent for cell proliferation in HBE cells, however further investigation must be performed in CFBE cells to investigate whether this channel is also important in this process, or if it simply is not involved in cell proliferation in CF airway epithelial cells.

In CFBE cells, $K_v10.1$ appears to play a role in cell proliferation since astemizole decreased cell number; however this inhibition was not seen in HBE cells (Figure 7). This finding is in agreement with previous reports in MCF-7 cells (Roy *et al*., 2008), which also showed that cell proliferation was significantly reduced in the presence of astemizole. Since $K_v10.1$ mRNA was not expressed in HBE cells, it is not surprising that astemizole did not decrease cell proliferation in this cell line. Therefore it is likely that there is a role for $K_v10.1$ in cell proliferation in CFBE cells. The same study by Roy and colleagues also reported that $K_v11.1$ did not play a role in cell proliferation when it was inhibited by E-4031 and in this study, treating HBE and CFBE cells with this $K_v11.1$ inhibitor had no affect on cell proliferation (Figure 7). Therefore, perhaps this channel is

not as important in cell proliferation as $K_v10.1$, and as I will discuss later in this chapter, it may be more crucial to other physiological processes pertaining to airway epithelial cells.

4.2.3 The role of $K_{Ca}1.1$ and $K_{Ca}3.1$ channels in HBE and CFBE cell proliferation.

Finally, the $K_{Ca}1.1$ inhibitor paxilline did not have any significant effect on cell proliferation in either cell line, while TRAM-34, which inhibits $K_{Ca}3.1$ channel activity, significantly decreased cell proliferation in HBE and CFBE cells (Figure 7). Previous literature from both MCF-7 (Roy *et al*., 2010) and NuLi cell lines (Trinh *et al*., 2008) both concluded this same result for TRAM-34. However, the results for the NuLi cell line, another model of bronchial epithelial cells, were again in combination with two other K^+ channel inhibitors, therefore it is impossible to determine precisely which channels are playing a role. However, it is unlikely that $K_v7.1$ is important in cell proliferation in their study (Trinh *et al*., 2008) because they used a non-selective $K_v7.1$ inhibitor (clofilium) and I believe that $K_{Ca}3.1$ is more important to cell proliferation in NuLi and CuFi cell lines, which is in agreement to what I have shown in HBE and CFBE cells.

4.2.4 Limitations in the selectivity of K^+ channels for cell proliferation studies.

A major drawback when studying the role of K^+ channels in cell proliferation relates to the specificity of each K^+ channel antagonist, meaning that when higher concentrations of inhibitors are used there is a greater chance that they may be acting on different transport proteins. For example, 293B selectively inhibits $K_v7.1$ channels when used at a specific IC_{50} value; however increasing this concentration also causes this antagonist to inhibit CFTR channels (Bachmann *et al*., 2001). When experiments are performed to investigate ion channel currents within the cell, the IC_{50} values are usually measured to determine the concentration of antagonist that will provide 50% inhibition of a particular channel, in order to be confident that the correct channel is under examination. However, unlike the study of ion channel currents, cell proliferation assays require much higher concentrations of inhibitors in order to elicit an effect (Wonderlin and Strobl, 1996). This is likely due to the addition of serum to the culture medium

during proliferation experiments, but not to the solutions used during current recordings. It has been suggested that the inhibitory drugs in question may bind to the serum in the culture medium, causing a decrease in the overall potency of the inhibitor (Wonderlin and Strobl, 1996). Since HBE and CFBE cells were treated in culture media containing 1% serum, due to the fact that treating them in serum-free media for 48 hr caused the cells to die, this is the main reason why the concentrations I chose for the cell proliferation experiment were much higher than their IC_{50} values shown in Table 2. Previous literature reports and concentration-response experiments were also used to select the optimal concentrations of pharmacological inhibitors for these studies, and from these results I used the lowest dose that produced a significant response as compared to controls. Although this approach is not perfect, when culture conditions were kept consistent it created significant and reproducible results, and was used as a guide to measure cell proliferation.

4.2.5 Proposed mechanism for the role of K^+ channels in cell proliferation in airway epithelial cells.

Although it has been concluded that hyperpolarization of the cell through the efflux of K^+ ions drives the cell through the G_1 phase of the cell cycle, the exact mechanism behind how this hyperpolarization can directly cause cells to proliferate is still not completely clear. I propose a model in Figure 19 in which the potential contribution of various K^+ channels is suggested to regulate the cell cycle and induce cell proliferation. These K^+ channels include members of the K_v7 family, $K_v4.1$ and $K_{Ca}3.1$ in HBE cells, as well as these channels and potentially $K_v10.1$ in CFBE cells. This model begins with the efflux of K^+ ions through activated K^+ channels, which then causes the cell membrane to become hyperpolarized. Activation of these K^+ channels may occur through an increase in $[Ca^{2+}]_i$, cAMP activity, and/or the involvement of mitogens. This hyperpolarization then creates a driving force for Ca^{2+} ions to enter the cell through open Ca^{2+} channels on the cell membrane. This influx of Ca^{2+} increases the $[Ca^{2+}]_i$, which in turn increases the activity of Ca^{2+}-dependent kinases within the cell, including Ca^{2+}/calmodulin-dependent kinases. These kinases regulate the activity of cyclins and cyclin-dependent kinases, which not only allow the cell to progress through G_1, but can also regulate cell cycle progression at various transition checkpoints. Therefore,

inhibiting these K^+ channels depolarizes the cell membrane, which can disrupt cell cycle progression (Wonderlin and Strobl, 1996). Although this is just a proposed mechanism, it may suggest how K^+ channel activity relates to cell proliferation in normal and CF bronchial epithelial cells, which can provide further insight into the mechanics involved in epithelial wound repair.

4.3 The activity of K^+ channels in mitogenic- and activator-stimulated cell proliferation.

Although one of the main objectives of this project was to study the role that K^+ channels played in basal cell proliferation, I was also interested in discovering any novel roles that various mitogens or K^+ channel activators might play in cell proliferation within HBE and CFBE cells. Moreover, if these potential mitogens and activators do affect cell proliferation, how was K^+ channel activity involved? Using a Coulter Counter to measure cell proliferation, I chose to investigate the mitogens EGF, IGF-1 and E2, as well as the K_v7 channel activator flupirtine maleate due to their implications in a variety of previous literature described below, in attempt to find a stimulator for cell proliferation in HBE and CFBE cells.

4.3.1 The role of mitogen-stimulated cell proliferation in HBE and CFBE cells.

In order to investigate the role of K^+ channels in mitogen-stimulated cell proliferation in HBE and CFBE cells, I first exposed cells to EGF for 48 hr and found that there was no significant increase in cell proliferation (Figure 9). Although it has been previously shown that EGF stimulates cell proliferation in a variety of cells types, including esophageal and prostatic epithelial cells, and granulosa cells (Gospodarowicz *et al*., 1977; Ilio *et al*., 1995; Rahman *et al*., 2010), it has also been shown that EGF does not affect cell proliferation in airway epithelial cells (Trinh *et al*., 2008). This group concluded that EGF is an important mediator of cell migration, but not cell proliferation, in the epithelial wound repair process in human airway epithelial cells (Trinh *et al*., 2008). Therefore perhaps EGF is a potent mitogen in some cell systems and not in others. However, even when not playing a role in cell proliferation in a particular cells system, perhaps its still involved in other physiological processes in that cell.

I was also interested in IGF-1 and E2, which are reportedly potent mitogens that play important roles in mediating the cell cycle in various cell types, including smooth muscle cells and mammary epithelial cells (Brooks and Skafar, 2004; Pluteanu and Cribbs, 2011). Airway epithelial cells express the IGF-1 receptor (IGF-1R) and this mitogen has been reported as playing a role in airway remodeling (Chihara *et al*., 1996). These cells also express the E2 receptor, ERα, and this mitogen is involved in ion transport within the lungs (Coakley *et al*., 2008). I found however that in HBE and CFBE cells, these mitogens did not increase cell number, suggesting that perhaps they simply are not important mediators of cell proliferation in human bronchial epithelial cells (Figure 9). Further investigation into whether these potential mitogens are involved in processes important to airway epithelial cells must occur and may be useful in understanding the mechanisms behind epithelial wound repair and CF.

4.3.2 The role of K_v7 channel activator-stimulated cell proliferation in HBE and CFBE cells.

The putative K_v7 channel activator flupirtine maleate was then used to examine HBE and CFBE cell proliferation, as it has been previously reported that it plays a role in K_v7 channel activation in the vascular tone of murine blood vessels (Yeung *et al*., 2007). I found however, that this potential activator did not increase cell proliferation in either cell line (Figure 10). Two potential interpretations were concluded from this experiment; firstly, perhaps flupirtine maleate simply does not activate K_v7 channels in human bronchial epithelial cells, or secondly, perhaps the activation of K_v7 channels in these cell lines does not have a direct effect on cell proliferation. Previous literature has also reported that when airway epithelial wound repair was studied in NuLi and CuFi cells, the K_{ATP} channel activator pinacidil did not increase cell proliferation, although it did increase cell migration in both cell lines (Trinh *et al*., 2008). Therefore, although the inhibition of K^+ channels causes a significant decrease in bronchial epithelial cell proliferation, perhaps the activation of these channels does not directly cause an increase in cell number. However further studies would be useful to determine if this is true for all K^+ channels in HBE and CFBE cells lines, by using different potential K^+ channel activators.

4.4 The activity of K^+ channels in the regulation of HBE cell volume in times of hyposmotic stress.

Another important function of airway epithelial cells is their ability to regulate their volume in times of altered intracellular and extracellular osmolarities. Cell volume regulation is a very important physiological process, and is related to many other airway epithelial cell functions, including ion transport and cell proliferation (Lang *et al*., 1998). In airway epithelial cells, disruptions in the osmolarity of the PCL volume can lead to changes in cell volume, which occur in order to maintain a state of homeostasis (Lock and Valverde, 2000). Cell volume must also double as the cell progresses through the cell-cycle so that after mitosis the daughter cells are able to be the same size as their original parent cell (Wang *et al*., 2002b). Therefore, cell volume regulation is very important and must be further examined in order to increase the understanding of normal and pathophysiological conditions concerning airway epithelial cells.

In times of hyposmotic stress, cells swell in size due to a fast influx of water that attempts to equilibrate the intra- and extracellular osmolarities. This process activates K^+ and Cl^- channels on the cell membrane and through the subsequent efflux of these ions, the cell will shrink back down to its original size which is defined as the RVD response (Lang *et al*., 1998). The activation of these K^+ channels in the regulation of cell volume has been studied in other cell systems, including Calu-3 and MCF-7 cells (vanTol *et al*., 2007; Harron *et al*., 2009), however in human bronchial epithelial cells it is not entirely known as to which K^+ channels are specifically involved in this process. Using the cell volume protocol explained in Section 2.9, my results show that HBE cells do elicit an RVD response when exposed to hyposmotic stress, which is in agreement with previous reports that demonstrated the involvement of this cell line in the regulation of cell volume and the RVD process (Fernandez-Fernandez *et al*., 2002). However, in the experiments by Fernandez-Fernandez and colleagues, they exposed cells to hyposmotic osmolarities of 215 mOsM, which did not elicit a measurable RVD response in my HBE cells (Figure 11). The only measurable response I saw was when HBE cells were exposed to 180 mOsM solutions, and therefore I used this osmolarity for all further experiments to elicit a measurable and reproducible RVD response. When pharmacological inhibitors were applied to the 180 mOsM hyposmotic solution, it was shown that the K_v and K_{Ca} channels that played an important role in the RVD response in HBE cells were K_v7.1,

$K_v11.1$, $K_{Ca}1.1$ and $K_{Ca}3.1$. The inhibitor concentrations that were used for these experiments were similar to the specific inhibitors IC_{50} value, since the experimental protocol for cell volume is much more similar to that performed to measure current recordings, than cell proliferation is, due to the fact that serum is not present and since the acute action of the K^+ channels of interest are being investigated rather than their chronic action, which was examined in the previous cell proliferation studies.

4.4.1 The role of K_v7 channels in HBE cell volume regulation.

XE991 was used to assess the role of K_v7 family members in the RVD response in HBE cells, and it significantly decreased the % RVD in this cell line (Figure 12 and 13). Previous reports have also shown that XE991 inhibits the RVD response in other cell types, including MCF-7 and Calu-3 cells (vanTol *et al*., 2007; Harron *et al*., 2009). I next wanted to further investigate which specific K_v7 channel member was involved, and used chromanol 293B, which significantly inhibited the RVD response in HBE cells (Figure 13). Unlike in Calu-3 cells, where $K_v7.1$ did not have an effect (Harron *et a*l., 2009), the inhibition of the RVD response by 293B has been seen in MCF-7 cells (vanTol *et al*., 2007), as well as in murine tracheal cells (Lock and Valverde, 2000). Although it was still not clear whether $K_v7.1$ is the sole K_v7 channel involved in the RVD response, the similarity between the reduction in % RVD between XE991 and 293B suggests that it indeed is. To further investiate this I used molecular knockdown experiments. I initially had hoped to test this hypothesis using siRNA techniques; however, after several months of unsuccessful attempts to produce a decrease in protein expression for $K_v7.1$ and $K_v7.5$ with siRNA (Figure 14), I decided to use a dominant-negative mutation to solve this problem. Before this, I first exposed HBE cells to hyposmotic solutions that contained both XE991 and 293B together and found that there was no additive effect on the % RVD, pointing towards the role for $K_v7.1$ in the RVD response (Figure 15). I then used a truncated isoform of $K_v7.1$, in which the NH_2-terminal end and the first one-third of the S1 segment was removed (Jiang *et al*., 1997; vanTol *et al*., 2007). These experiments demonstrated that when HBE cells were transfected with this truncated $K_v7.1$ isoform (pIRES2-EGFP-ΔN-$K_v7.1$) and exposed to hyposmotic conditions, they failed to elicit a measurable RVD response compared to the pIRES2-EGFP vector control (Figure 16).

From these results, I conclude that K_v7.1 is primarily involved in the RVD response in HBE cells. Although K_v7.2, 7.4 and 7.5 are also expressed in HBE cells, without adequate experiments that target these channels specifically, their potential role in the RVD response cannot be examined. Since murine K_v7.5 channels alone have been implicated in changes in cell volume in *Xenopus* oocytes (Jensen *et al*., 2005), this member of the K_v7 channel family may play an important role in HBE cells. Because K_v7.5 siRNA studies were unsuccesful, future experiments using a dominant-negative variant of K_v7.5 could be useful to describe the role that this channel plays in the regulation of HBE cell volume and the RVD response. Recently, mice models that contain a dominant negative mutation in the K_v7.5 pore have been created (Tzingounis *et al*., 2010), and hopefully this is only the beginning in the investigation into this less understood K^+ channel.

4.4.2 The role of K_v4, K_v10 and K_v11 channels in HBE cell volume regulation.

I next looked at the role that K_v4.1 and K_v4.2 play in the RVD response, since it has been previously reported that K_v4.1 is an important player in the RVD process in Calu-3 cells (Harron *et al*., 2009). This group also suggested that K_v4.2 did not play an important role in this process (Harron *et al*., 2009). I discovered that in HBE cells, the pharmacological inhibition of K_v4.1 and K_v4.2 by flecainide and stromatoxin respectively did not affect the RVD response compared to controls (Figure 12 and 13). Since I saw no effect of K_v4.2 in cell proliferation either, this channel is clearly involved in other respiratory epithelial processes, perhaps similar to those proposed in alveolar epithelial cells, such as K^+ recycling into the lumen (Lee *et al*., 2003) and oxygen sensing (O'Grady and Lee, 2005). A second explanation could be that since there is no role for this channel in either of these two physiological processes and protein expression could not be examined in HBE cells, perhaps K_v4.2 is not truly expressed in this cell line. On the other hand, K_v4.1 does presumably play a role in cell proliferation; therefore although its protein expression was not examined it does seem to have a functional role in HBE cells, however not in terms of regulating the RVD response.

I next wanted to look at the role K_v10.1 and K_v11.1 play in the RVD response. Although K_v10.1 mRNA was not expressed in HBE cells, I used astemizole to primarily

act as a positive control and found that the addition of astemizole (at concentrations near it's IC_{50} value) to the hyposmotic solution did not significantly affect the RVD response (not shown). This supported my finding that $K_v10.1$ is not expressed in HBE cells. I next wanted to test whether astemizole can also inhibit $K_v11.1$ channels, since it has been reported that a much lower IC_{50} value can also inhibit this channel (Cavalli *et al*., 2002). I again found that this did not significantly affect the RVD response in HBE cells (Figure 13), which suggests that astemizole is perhaps more selective towards $K_v10.1$ than $K_v11.1$. Unlike $K_v10.1$, $K_v11.1$ mRNA is expressed in HBE cells, and has been previously implicated in playing a role in the RVD response in MCF-7 cells (Roy *et al*., 2008). Using the same pharmacological inhibitor for $K_v11.1$, E-4031 that was used by Roy and colleagues, I found that in HBE cells it also reduced the % RVD (Figure 12 and 13). Therefore, I conclude a role for $K_v11.1$ in the regulation of cell volume in times of hyposmotic stress in HBE cells.

4.4.3 The role of $K_{Ca}1.1$ and $K_{Ca}3.1$ channels in HBE cell volume regulation.

I finally investigated the role that $K_{Ca}1.1$ and $K_{Ca}3.1$ play in the RVD response. It has been previously reported that $K_{Ca}1.1$ and $K_{Ca}3.1$ mediate the RVD response in bronchial and tracheal epithelial cells respectively (Vazquez *et al*., 2001; Fernandez-Fernandez *et al*., 2002). My results confirmed these previous reports by demonstrating that $K_{Ca}1.1$ did play a role in the RVD response, since pharmacological inhibition of this channel with paxilline caused a significant reduction in the % RVD (Figure 12 and 13). I also found that inhibiting $K_{Ca}3.1$ with TRAM-34 also significantly reduced the % RVD (Figure 13). Although $K_{Ca}3.1$ is primarily involved in the RVD response of tracheal epithelial cells, perhaps it is also important in HBE cells. There is a smaller reduction in % RVD when TRAM-34 was used than there is for paxilline, therefore perhaps $K_{Ca}3.1$ is playing a less important role than $K_{Ca}1.1$ in mediating cell volume in times of hyposmotic stress in HBE cells, which would support the findings by Vazquez and Fernandez-Fernandez.

4.4.4 Limitations in the study of K^+ channels and the RVD response in HBE cells.

Unlike the limitations that were described for the study of K^+ channels and cell proliferation in relation to the selectivity of the K^+ channel inhibitors, the selectivity for the antagonists used in the study of cell volume and the RVD response closely reflect the IC_{50} values of the inhibitors involved. Since the cells are perfused in solutions that do not contain serum, the higher inhibitor concentration was not needed. However, a major limitation to this study, which is also pertinent to the cell proliferation study, is the inability to complete successful siRNA experiments. Without siRNA targeting, my conclusions are not as strong, and questions continue to arise about whether the pharmacological findings are truly real because direct targeting of a channel at the molecular level is much more selective that at the pharmacological level. Another limitation, and one that continuously arises when using model cell lines, is how representative the model cells are to native human bronchial epithelial cells. Cultured cells can lose particular phenotypes specific to native cells (Ehrhardt *et al*., 2002, 2005) However as mentioned above, these cell lines do contain many of the same characteristics as native cells and have been shown to be close representative models to their native cell counterparts. Finally, the expression of accessory subunits, including members of the minK family, may alter the findings for both cell proliferation and cell volume regulation; however, I did not investigate the expression of these subunits in HBE or CFBE cell lines. For example, the IC_{50} value for XE991 is higher for $K_v7.1$ channels that co-assemble with minK proteins (Wang *et al*., 2000), therefore it must be determined if this co-assembly between $K_v7.1$ and minK occurs in bronchial epithelial cells. Co-immunoprecipitation could then be performed if a co-assembly is discovered, in order to better understand the molecular nature of these complement proteins.

4.4.5 Proposed mechanism for the role of K^+ channels in cell volume regulation and the RVD response in HBE cells.

The mechanism behind how HBE cells elicit their RVD response is not completely clear; however, there are different theories as to how this occurs. The most relevant theory involves a particular role for Ca^{2+} in order to initiate this response and is shown in Figure 20. In human bronchial epithelial cells, it was reported that an increase in $[Ca^{2+}]_i$ was observed after hyposmotic cell swelling (Fernandez-Fernandez *et al*.,

2002), and was then suggested that TRPV4, a non-selective cation channel may be the entry pathway for Ca^{2+} into the cell, which then activates $K_{Ca}1.1$ channels, which are responsible for mediating the RVD response in bronchial epithelial cells (Fernandez-Fernandez *et al*., 2002; Becker *et al*., 2005). Since $K_{Ca}3.1$ also seems to be playing a role in my cell line, this increase in $[Ca^{2+}]_i$ may be activating this channel as well. Although I have not personally investigated TRPV4 in HBE or CFBE cells, its expression has been previously reported in both cell lines (Fernandez-Fernandez *et al*., 2008). This activation of K_{Ca} channels allows for the efflux of K^+ ions from the cell, initiating the RVD response. This efflux of K^+ ions would then hyperpolarize the cell membrane and create a driving force for Ca^{2+} entry into the cell through open Ca^{2+} channels. This would then increase the $[Ca^{2+}]_i$ and activate K_{Ca} channels like $K_{Ca}1.1$ and $K_{Ca}3.1$, and also cause the membrane to depolarize, activating K_v channels like $K_v7.1$ and $K_v11.1$ on the cell membrane which would all cause the efflux of K^+ ions and create the RVD response. It has been shown that in tracheal epithelial cells, when TRPV4 was knocked down, it abolished the $K_{Ca}3.1$ current involved in mediating the RVD response in these cells (Arniges *et al*., 2004). This experiment was also performed in HBE cells (Fernandez-Fernandez *et al*., 2008) and showed a decrease in $K_{Ca}1.1$ current when TRPV4 was knocked down. This strongly suggests that the candidate for Ca^{2+} influx into these cells is TRPV4, and further investigation into the specific signaling mechanism that occurs once Ca^{2+} has entered the cell and leads to the RVD response must be performed.

4.5 The regulation of CFBE cell volume regulation in times of hyposmotic stress.

I next wanted to investigate whether CFBE cells elicited an RVD response. Previous literature has suggested that these cells do not undergo the RVD process due to their dysfunctional CFTR protein (Vazquez *et al*., 2001), however I wanted to use extreme hyposmotic shock to examine if these reports had just not tried low enough osmolarities to induce a response. However, I was unable to elicit an RVD response in 125 or 180 mOsM hyposmotic solutions (Figure 17). In human tracheal epithelial cells, it was shown that the K^+ channel mediating the RVD response was $K_{Ca}3.1$, and that although the CF human tracheal epithelial cells containing the homozygous ΔF508 CFTR mutation showed functional $K_{Ca}3.1$ activity, the channel was not activated in response to

hyposmotic-induced swelling (Vazquez *et al*., 2001). It is still not clear what the link is between CFTR and the K^+ channels involved in the RVD response, however multiple hypotheses have been suggested. First, it has been reported that ATP may relate the two, since ATP is released when cells are exposed to hyposmotic shock, and this release may be increased by CFTR through the efflux of intracellular Cl^- (Hazama *et al*., 2000). Although this is a possibility, it has yet to be proved as to how this is related to K^+ channel activation since this hypothesis is directed towards the activation of Cl^- channels and the RVD response (Vazquez *et al*., 2001). A second hypothesis is related to Ca^{2+}, and proposes that CFTR may be required for Ca^{2+} entry into the cell under times of hyposmotic stress (Reinlib *et al*., 1992). Although it is not exactly known as to how this occurs, it has been reported that there is a dysfunction in the activation of the K^+ channels involved in the RVD response via the swelling-mediated pathway (Arniges *et al*., 2004). The lack of functional CFTR in CF airway epithelial cells may have a direct or in-direct affect on the Ca^{2+} entry protein in HBE cells, since CFTR is able to modulate other transport proteins through changes in post-transitional regulation or protein expression (Schwiebert *et al*., 1999; Kunzelmann, 2001) This hypothesis has been expanded upon, and it is currently thought that there is a dysfunction in the Ca^{2+} entry into the cell via TRPV4 in CF airway epithelial cells (Arniges *et al*., 2004). Arniges and colleagues showed that both tracheal and bronchial epithelial cell lines did not RVD in the presence of hyposmotic stress and also did not display an increase in $[Ca^{2+}]_i$ when exposed to these conditions. These cells did however express TRPV4 mRNA, and when TRPV4 was activated by 4α-phorbol 12, 13-didecanoate (4α-PDD), the RVD response was restored as well as the $[Ca^{2+}]_i$. Therefore, they concluded that there is some sort of dysfunction in the TRPV4 channel in CF cells that does not allow for adequate Ca^{2+} influx, causing K_v and K_{Ca} channels to not activate and elicit an RVD response. It has been proposed that stimuli that cause an increase in $[Ca^{2+}]_i$, such as cell swelling by hyposmotic stress, causes an decrease in Na^+ absorption (Arniges *et al*., 2004). Therefore the decrease in induced Ca^{2+} signal due to a dysfunction in the TRPV4 channel may lead to an increase in Na^+ absorption, a hallmark of CF. This work may one day help better understand the consequences of hyposmotic stress on CF airway epithelial cells .

4.6 The regulation of HBE and CFBE cell volume regulation in times of hyperosmotic stress.

Although one of the main objectives of my project was to investigate the role of K^+ channels in the RVD response, I was also interested in determining whether HBE and CFBE cells were involved in regulating their cell volume in times of hyperosmotic stress. In this process, cells shrink in size due to the efflux of water that attempts to equilibrate the intra- and extracellular osmolarities. The cell then swells back up to original size, known as an RVI response. It has been previously reported that Calu-3 cells do not undergo this RVI response, because unlike the activation of K^+ and Cl^- channels that are involved in the RVD process, the RVI response activates $Na^+/K^+/2Cl^-$ co-transporters, Na^+/H^+ exchangers and some Na^+ channels (Lang *et al*., 1996; Harron *et al*., 2009). Since Calu-3 cells are mainly involved in Cl^- secretion, and do not express functional ENaC, it is not unexpected that these cells do not undergo an RVI response (Harron *et al*., 2009). However, since HBE and CFBE cells do play a role in Na^+ absorption, they may elicit an RVI response in times of hyperosmotic stress. However, contrary to my hypothesis, I found with varying degrees of hyperosmotic shock, neither HBE nor CFBE cells elicited this response (Figure 18). Therefore, further examination behind the mechanisms of the RVI response must be performed to fully understand this process, and perhaps the ability of HBE and CFBE cells to play a role in Na^+ absorption does not mean that they are involved in every physiological process mediated by Na^+ transport proteins.

4.7 The significance of K^+ channel activity in cell proliferation and cell volume regulation in respiratory epithelial physiology.

A better understanding of the functions of airway epithelial cells will allow us to gain further insight into how they are involved in human physiology. This study investigated how two human airway epithelial cell lines are involved in cell proliferation and cell volume regulation, and more specifically, how K^+ channels affect these physiological processes. Although this is just an investigation on 9 of the approximately 40 K^+ channels that are in the airway epithelium, it is an attempt to begin delineating the role of these channels in this system. It is also an informative comparison as to how normal and CF bronchial epithelial cells differ in not only their K^+ channel physiology, but also in physiological processes that are important to their functioning. It is my hope

that there will be future reports on K^+ channel involvement in the airways, and that this work will help to better characterize their place in human physiology and perhaps lead the way for potential therapeutic K^+ channel targeting for airway diseases such as CF.

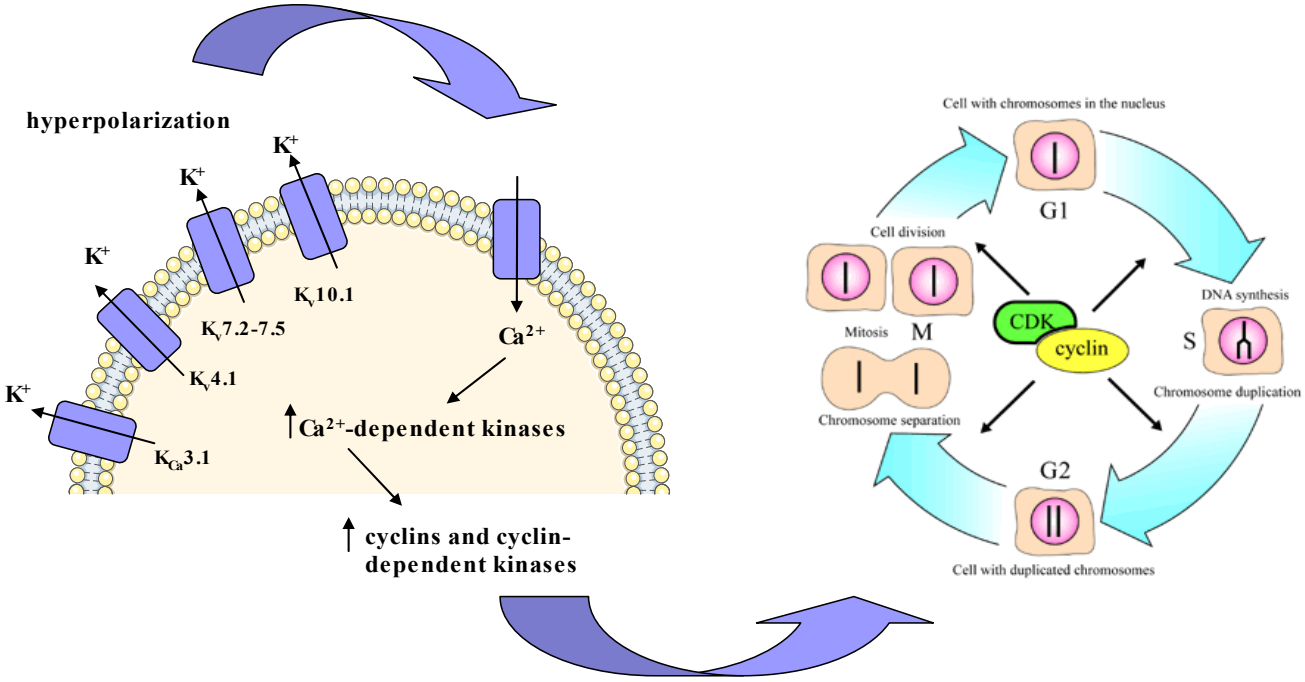


Figure 19. Schematic model illustrating the mechanisms involved in K^+ channel mediated cell proliferation in HBE and CFBE cells. The efflux of K^+ ions through activated K^+ channels, including K_v7.2-7.5, K_v4.1, K_v10.1 and K_{Ca}3.1, causes the cell membrane to become hyperpolarized. Next, this hyperpolarization creates a driving force for Ca^{2+} entry into the cell through open Ca^{2+} channels on the cell membrane. This influx of Ca^{2+} increases the $[Ca^{2+}]_i$, which in turn increases the activity of Ca^{2+}-dependent kinases within the cell, including Ca^{2+}/calmodulin-dependent kinases. These kinases regulate the activity of cyclins and cyclin-dependent kinases, which not only allow the cell to progress through G_1, but can also regulate cell cycle progression at various transition checkpoints (Wonderlin and Strobl, 1996; Hartwell *et al*., 2001).

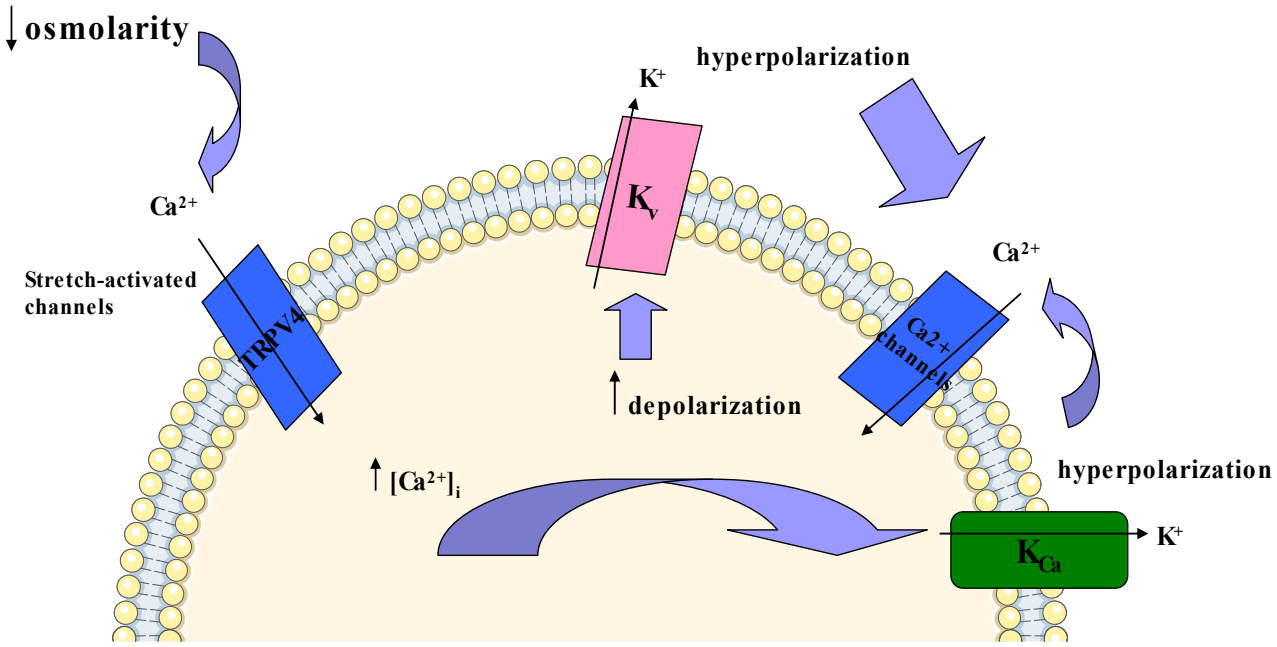


Figure 20. Schematic model illustrating the mechanisms involved in K^+ channel mediated cell volume regulation in HBE cells. A decrease in extracellular osmolarity activates stretch-activated channels, presumably TRPV4, which causes the influx of Ca^{2+} into the cell. The increase in $[Ca^{2+}]_i$ can then activate $K_{Ca}1.1$ and $K_{Ca}3.1$ in HBE cells, which expel K^+ ions and cause the RVD response. This efflux of K^+ ions will hyperpolarize the cell membrane, creating the driving force for Ca^{2+} to enter the cell through open Ca^{2+} channels. This creates more $[Ca^{2+}]_i$, which depolarizes the cell membrane and activate K_v channels such as $K_v7.1$ and $K_v11.1$, which expel more K^+ ions and contribute to the RVD response (Lang *et al*., 1996; Fernandez-Fernandez *et al*., 2002).

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APPENDIX A: TABLES

Table 1. K^+ channel name and their corresponding gene name.

Channel Name	Gene Name
$K_v7.1$	KCNQ1
$K_v7.5$	KCNQ5
$K_v4.1$	KCND1
$K_v4.2$	KCND2
$K_v4.3$	KCND3
$K_v10.1$	KCNH1
$K_v11.1$	KCNH2
$K_{Ca}1.1$	KCNMA1
$K_{Ca}3.1$	KCNN4

Table 2. K^+ channel inhibitors and information.

Drug Name	Channels Inhibited	Vehicle	Stock [C]	IC_{50} value	Company
XE991	K_v7.1-7.5	H_2O	100 mM	0.6-1 μM (Tocris)	Tocris Bioscience
Chromanol 293B	K_v7.1	Dimethyl Sulfoxide (DMSO)	100 mM	1-10 μM (Tocris)	Tocris Bioscience
Flecainide	K_v4.1-4.3	Methanol	50 mM	11-100 μM (Barfield *et al*., 2005; Amberg *et al*., 2002)	Sigma-Aldrich
Stromatoxin	K_v4.2	H_2O	20 μM	1.2 nM (Escoubas *et al*., 2002)	Sigma-Aldrich
Astemizole	K_v10.1	DMSO	100 mM	200 nM (Garcia-Ferreiro *et al*., 2004)	Sigma-Aldrich
E-4031	K_v11.1	H_2O	2 mM	14 nM (Yao *et al*., 2005)	Sigma-Aldrich
Paxilline	K_{Ca}1.1	Chloroform	10 mM	1.9 nM (Tocris)	Sigma-Aldrich
TRAM-34	K_{Ca}3.1	DMSO	14.5 mM	20 nM (Tocris)	Sigma-Aldrich

Table 3. PCR primer sequences and information.

Channel Name	Primer Sequences	Annealing Temp (°C)	Base Pair Size (bp)	Reference
K_v7.1	**Forward** 5'-CTTGCCATCTCTTCTTG-3' **Reverse** 5'-AGTGTTGGGCTCTTCCTTAC-3'	HBE: 56 CFBE: 60	411	Moser *et al*., 2008
K_v7.5	**Forward** 5'-CGCTTTCGTTTTTCTCCTTG-3' **Reverse** 5'-CGAGCAAACCTCAGTCTTCC-3'	HBE: 59 CFBE: 60	207	Moser *et al*., 2008
K_v4.1	**Forward** 5'-CCACTTGCTGCAGTGTCTAG-3' **Reverse** 5'-TGTCGCAGTTCAGGTCAAGG-3'	HBE: 65 CFBE: 65	335	Bai *et al*., 2007
K_v4.2	**Forward** 5'-ATCTTCCGCCACATCCTGAA-3' **Reverse** 5'-GATCCGCACGGCACTGTTTC-3'	HBE: 59 CFBE: 59	362	Bai *et al*., 2007
K_v4.3	**Forward** 5'-GATGAGCAGATGTTTGAGCAG-3' **Reverse** 5'-AGCAGGTGGTAGTGAGGCC-3'	Calu-3: 60	106	Bai *et al*., 2007
K_v10.1	**Forward** 5'-CGCATGAACTACCTGAAGACG-3' **Reverse** 5'-TCTGTGGATGGGGCGATGTTC-3'	CFBE: 60	479	Borowiec *et al*., 2007
K_v11.1	**Forward** 5'-ATGTGACGGCGCTCTACTTC-3' **Reverse** 5'-GAGTACAGCCGCTGGATGAT-3'	HBE: 60 CFBE: 60	172	Roy *et al*., 2008
K_{Ca}1.1	**Forward** 5'-GGAATGGGAGACGCTTCATA-3' **Reverse** 5'-CCTGCAGCGAAGTATCATCA-3'	HBE: 60 CFBE: 60	153	Roy *et al*., 2009
K_{Ca}3.1	**Forward** 5'-CATCACATTCCTGACCATCG-3' **Reverse** 5'-ACGTGCTTCTCTGCCTTGTT-3'	HBE: 60 CFBE: 60	158	Brakemei *et al*., 2003
HPRT	**Forward** 5'-GCCAGACTTTGTTGGATTTG-3' **Reverse** 5'-CTCTCATCTTAGGCTTTGTATTTTG-3'	HBE: 60 CFBE: 60	141	Roy *et al*., 2006

Table 4. Antibody information.

Antibody	Channel Name	Protein Size (kDa)	Host	Dilution	Company
Primary (1°)	$K_v7.1$	75	Rabbit	1:500	Santa Cruz Biotechnology, INC.
	$K_v7.5$	116	Rabbit	1:2000	Millipore
Secondary (2°)	Anti-Rabbit IgG	N/A	Goat	1:2000	Cell Signaling Technology

APPENDIX B: RECIPES

Fibronectin coating solution (100 mL)
97.8 mL LHC basal medium
0.2 mL BSA (1 mg/ml)
1 mL Vitrogen 100
1 mL Human fibronectin (1mg/ml)

Hyperosmotic bath solution (1 L)
4.383 g NaCl
0.186 g KCl
0.176 g CaCl2
0.1016 g MgCl2
0.901 g Glucose
2.383 g Hepes
Mannitol varies for different osmolarities
900 mL ddH_2O
Stir until completely dissolved
Bring up to 1 L with ddH_2O; pH to 7.4

Hyposmotic bath solution (1 L)
4.383 g NaCl
0.186 g KCl
0.176 g CaCl2
0.1016 g MgCl2
0.901 g Glucose
2.383 g Hepes
Mannitol varies for different osmolarities
900 mL ddH_2O
Stir until completely dissolved
Bring up to 1 L with ddH_2O; pH to 7.4

Isosmotic bath solution (1 L)
4.383 g NaCl
0.186 g KCl
0.176 g CaCl2
0.1016 g MgCl2
0.901 g Glucose
2.383 g Hepes
27.32 g Mannitol
900 mL ddH_2O
Stir until completely dissolved
Bring up to 1 L with ddH_2O; pH to 7.4

Phosphate buffered saline (PBS) (10X, 1 L)
13.40 g $Na_2HPO_4 \cdot 7H_2O$
8.0 g NaCl
0.2 g KCl
0.24 g KH_2PO_4
900 mL ddH_2O
Stir until completely dissolved
Bring up to 1 L with ddH_2O; pH to 7.4

Resolving gel (7.5%, 1 gel)
3.575 mL ddH_2O
1.875 mL 30% Acyrlamide mix
1.9 mL 1.5 M Tris (pH 8.8)
0.075 mL 10% SDS
0.075 mL 10% APS
0.006 mL TEMED
Add APS and TEMED immediately before pouring the gel

RIPA buffer
4 mg Deoxycholic acid
5 mL Triton-X-100
5 mL SDS
4.38 g NaCl
12 mL 1mM EDTA
0.606 g Tris-HCl
Stir until completely dissolved; pH to 7.4

Running buffer (10X, I L)
700 mL ddH_2O
144 g Glycine
30.3 g Tris base
10 g 1% SDS
Stir until completely dissolved; bring up to 1 L with ddH_2O

Stacking gel (5%, 1 gel)
3.35 mL ddH_2O
1.0 mL 30% Acyrlamide mix
1.5 mL 0.5 M Tris (pH 6.8)
0.060 mL 10% SDS
0.060 mL 10% APS
0.006 mL TEMED
Add APS and TEMED immediately before pouring the gel

TBS (10X, 1 L)
700 mL ddH_2O
292.2 g NaCl
24.22 g Tris base
Bring up to 1 L with ddH_2O

TBS-T (1 L)
100 mL 10X TBS
890 mL ddH_2O
10 mL 10% Tween

Transfer buffer (10X, 1 L)
700 mL ddH_2O
144 g Glycine
30.3 g Tris base
1.0 g 1% SDS
Stir until completely dissolved; bring up to 1 L with ddH_2O

Tris boric acid EDTA (TBE) (10X, 1 L)
108 g Tris base
55 g Boric acid
40 mL 0.5M EDTA
800 mL ddH_2O
Stir until completely dissolved
Bring up to 1 L with ddH_2O