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REGULATION OF Cl^-/HCO_3^- TRANSPORT AND
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REGULATION OF Cl^-/HCO_3^- TRANSPORT AND EXTRACELLULAR pH BY CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR

By

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ABSTRACT

REGULATION OF Cl^-/HCO_3^- TRANSPORT AND EXTRACELLULAR pH BY CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR
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The disease, cystic fibrosis (CF), is caused by the malfunction of the cystic fibrosis transmembrane conductance regulator (CFTR). Expression of functional CFTR may normally regulate extracellular pH via control of HCO_3^- efflux. Recent reports even suggest that the CFTR may be a Cl^-/HCO_3^- exchanger. This could be very important for treatment of CF, but is not well understood. Contained herein, I examined four possible models of CFTR function in the transport of HCO_3^-: 1) CFTR as a Cl^- channel; 2) CFTR as a Cl^-/HCO_3^- anion exchanger (AE); 3) CFTR as a Cl^- channel and AE; and 4) CFTR as a Cl^- channel with regulation of AE activity. In addition, I present two different approaches in the evaluation of these models; both, a presentation of data from my own research as well as a literature review of the research of others regarding current data on CFTR's function in transport of bicarbonate and regulation of pH. In my own experiments, the effect of stimulators and inhibitors of CFTR and AEs' via parallel studies of iodide (anion) efflux and changes in pH_o were conducted. My data, as well as that generated by other laboratories, indicate that even though CFTR does support and regulate HCO_3^- efflux in the cell lines examined, it is highly unlikely that the control observed was due to Cl^-/HCO_3^- exchanger-like transport.

To my parents, Ivan and Nada Krha.

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KEY TO SYMBOLS OR ABBREVIATIONS

CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
pH_o	Extracellular pH
pH_i	Intracellular pH
AE	Anion Exchanger
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DPC	Diphenylamine-2-dicarboxylate
DMSO	Dimethyl Sulfoxide
For	Forskolin
Glib	Glibenclamide
G418	Geneticin
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
EB	Efflux Buffer (no iodide)
LB	Loading Buffer (with iodide)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
C127	Mouse mammary epithelial cells
2WT2	C127 cells stably expressing wild type CFTR
508-8	C127 cells stably expressing mutated (ΔF508) CFTR
ΔF508	CFTR gene carrying a mutation at amino acid position 508 (deletion of phenylalanine)
WT	Wild type CFTR
BPV	C127 cells transfected with Bovine Papilloma-based Vector (control cells)
ENaC	Epithelial Na^+ channels
ROMK	Renal K^+ channels
AQP3	Aquaporin type III water channels
NHE3	Na^+/H^+ exchanger isoform 3

NHERF	Na^+/H^+ exchanger regulatory factors
NBC3	Na^+-HCO_3^- cotransporter 3
SLC26	Family of Cl^-/HCO_3^- exchangers.
DRA	Downregulated in adenoma
ASF	Airway Surface Fluid
ABC	ATP-binding cassette
NBD	Nucleotide Binding Domain (CFTR structure)
MSD	Membrane Spanning Domain (CFTR structure)
R Domain	Regulatory Domain (CFTR structure)
C1, C2	Closed states of CFTR
O1, O2	Open states of CFTR
PDZ domain	Protein-protein Interaction Domains (CFTR structure)
PKA	Protein Kinase A
cAMP	Cyclic Adenosine Monophosphate
$[cAMP]_i$	Intracellular concentration of cAMP
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
ER	Endoplasmic Reticulum
SMG	Submandibular Glands
VIP	Vasoactive Intestinal Peptide
CBAVD	Congenical Bilateral Absence of Vas Deferens
LAPS	Light-Addressable Potentiometric Sensor
LED	Light Emitting Diode
SEM	Standard Error of Mean
n	Trial number
P	Probability
t	Time

INTRODUCTION

1. Cystic Fibrosis (CF) Background

Cystic fibrosis (CF) is the most common fatal genetic disease affecting caucasians in the United States. CF is caused by the mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, found on the long arm of chromosome seven (Wine, 1991). In the United States, about 5% of individuals (one in 20) in the caucasian population are asymptomatic carriers (they posses one mutant allele) and about one in every 2,500 children born, contain both defective alleles and thus express the disease. In the United States this results in about 1,000 new cases of CF each year and about 30,000 individuals living with the disease (Welsh and Smith, 1995). CF patients were once not expected to survive past their early childhood (Quinton, 1999). With the increased understanding of the disease itself and treatments available, the pediatric mortality rate has been reduced, and presently a typical CF patient can expect to survive into their thirties (Quinton, 1999).

The number of individuals carrying the most common, specific mutation (ΔF508) in their CFTR gene is very high. Geneticists found this intriguing and often lay people find it difficult to understand why a lethal genetic disease would survive through natural selection (Rodman and Zamudio, 1991). It is thought that historically the defective allele may have been preserved and favored in the gene pool in Europeans (even more so in Eastern Europe) because it provided a selective advantage to carriers stricken by cholera (Rodman and Zamudio, 1991;

Thiagarajh and Verkman, 2003). CFTR is chiefly expressed in human epithelial cells, where it normally functions as a Cl^- channel and, as such, it also functions as a pathway that facilitates secretion of bodily fluids in endotoxin-mediated diarrheas, as is the case in cholera (Thiagarajh and Verkman, 2003). One hypothesis is that with the lack or reduced expression of CFTR (as in CF heterozygotes or carriers), the loss of fluid in such diseases is decreased, thus aiding in the survival by natural selection of the heterozygous individuals and supporting this environmentally based advantage (Rodman and Zamudio, 1991). Cholera studies with knockout CFTR mice support this hypothesis (Grubb and Gabriel, 1997). There also are reports that the selective advantage of CF heterozygosity is a result of higher human resistance towards *Mycobacterium tuberculosis* (Meindl, 1987). In this case, it is thought that the CF heterozygosity increased successful human survival in vast areas of Europe once affected by this disease (Meindl, 1987). This selective advantage maintains the continuous presence of various mutants in the population, but does not explain the high prevalence (about 90%) of the most common CFTR mutation (ΔF508) (Sferra and Collins, 1993). The founder effect (change in genetic variation due to a few pioneering individuals), however, could explain its initial and therefore consequential prevalence (Purves et al., 2004).

The cystic fibrosis gene has been cloned and sequenced, expressed in various cell lines, and its gene product, CFTR, has been shown to be a small conductance Cl^- channel regulated by cAMP found on the apical surfaces of epithelial cells (Riordan et al., 1989; Gray et al., 1989; Gregory et al., 1990;

Kartner et al., 1991; Tabcharani, et al., 1991; Bear et al., 1992). Other monovalent anions can pass through CFTR in addition to Cl^-, with a selective preference of $Br^- \geq Cl^- > I^- > F^-$ (Anderson et al., 1991b). It also has been shown that HCO_3^- ions pass through CFTR, even though the importance of such a function is not yet well characterized (Poulsen et al., 1994). The ΔF508 mutation of CFTR and consequent loss of Cl^- conductance can explain the pathophysiology of many organ systems altered in CF, where reduced Cl^- permeability leads to dehydrated and clogged ducts (Wine, 1991; Bear et al., 1992; Quinton, 1983; Welsh, 1990). All of the consequences leading to fatality in CF, however, have been difficult to rationalize solely as a defect in Cl^- secretion (Kunzelmann, 1999). The majority of CF patients (about 90%) will die of lung disease (Welsh and Smith, 1995). This appears to be a consequence of a high bacterial infection rate as a result of the thick mucus buildup and low clearing by cilia (Welsh and Smith, 1995). This buildup of mucus and preferential infection by certain pathogens (like *Staphylococcus aureus* and *Pseduomonas aeruginosa*) has been hard to explain solely by abnormal Cl^- transport and is not completly understood.

2. Pathophysiology of CF

The exact origin of CF is unknown, although, CF may have first occurred around 5,000 years ago in Eastern Europe and was further expanded in the population through human migration, gene mutations, and new diet conditions (Busch, 1990). Historical evidence of the disease was first recorded in folk stories

and diaries in Europe as early as the 1600s' (Busch, 1990). Children with the disease did not survive early childhood and as result were often thought to be "bewitched" if they had a salty forehead. These historical documents mention both the salty forehead in children, as well as some early autopsy results which note damage observed in the pancreas (Busch, 1990). In fact, the disease is sill commonly diagnosed by excessive salt in the sweat. Clinically, however, CF affects more organs than just the skin. It also affects the lungs, liver, pancreas, small intestine and reproductive tract. Overall, the disease is characterized by defective electrolyte transport and clogging of ducts with thick, sticky mucus. In all organs affected in CF, mutations in the CFTR gene directly lead to abnormal Cl^- permeability as well as indirectly to abnormalities in Na^+ and HCO_3^- balance (Quinton, 1983; Smith and Welsh, 1992; Seidler et al., 1997; Quinton, 1999; Shumaker et al., 1999; Ballard et al., 1999; Lee et al., 1999a; Ahn et al., 2001).

2.1. Observations on the Skin

The skin in CF is affected by a malfunction of sweat glands and electrolyte imbalance. This is also the only organ involved where thick mucus does not play a role (Quinton, 1999). Sweat glands in the skin normally secrete NaCl in the secretory coil of the sweat gland as an isotonic fluid (Quinton, 1999). Water, Na^+ and Cl^- are transported out of the body and into the gland across the membranes of the epithelial cells lining the secretory coil. This isotonic fluid (sweat) accumulates in the lumen of the coil, and then, as the sweat travels up through the absorptive duct on a way to the skin surface, Na^+ and Cl^- are reabsorbed

back into the body, making sweat hypotonic when it arrives at the skin surface. The reabsorption of Na^+ and Cl^- occurs through channels in the epithelial cells lining the absorptive duct (specifically epithelial Na^+ channels, ENaC, and CFTR respectively) (Reddy and Quinton, 2003). In a healthy individual the reabsorption of Cl^- is mediated through CFTR, while in CF this doesn't occur; the Cl^- stays in sweat. Interestingly, the conductance of Na^+ through ENaC in sweat glands appears to be associated with the function of CFTR in some way because in CF the reabsorption of Na^+ is also reduced (Reddy and Quinton, 2003). As a result, in diseased individuals, the lack of CFTR is manifest as an abnormally salty sweat (still isotonic) due to the defect in reabsorption in the sweat duct (Quinton, 1983). In the skin, the malfunctioning or absent CFTR Cl^- channel can reasonably serve as the sole and sufficient explanation of its pathophysiology in CF.

2.2. CF Effect in the Airway

The airway epithelium consists of surface epithelium and secretory submucosal glands. It is believed that in healthy individuals Na^+ and Cl^- are secreted out of epithelial cells in the submucosal gland and absorbed back into the cells on the surface epithelium of lung, thus maintaining proper electrolyte balance (Na^+ and Cl^-) and "healthy" airway surface fluid (Boucher et al., 1983; Jiang et al., 1993; Smith and Welsh, 1993; Smith et al., 1994; Smith et al., 1996). Na^+ and Cl^- are secreted by the submucosal gland as a mechanism to get water moving into the gland, which in turn will secrete salt, water and mucus. Na^+ is

absorbed back at the surface epithelium with Cl^- and excess water passively follows. Airway surface fluid (ASF) is defined as a thin layer of liquid that covers the lung surface epithelium (Smith et al., 1996). Some research groups believe that in the disease, as a result of the lack of CFTR on the lung surface epithelium and its regulation of the Na^+ channel (ENaC), Na^+ absorption is increased, while Cl^- absorption is decreased (Welsh and Smith 1995, Mall et al., 2004). It has been shown that, as a result of the abnormal electrolyte balance and transport, the ASF in CF patients has increased concentrations of salt and in particular Cl^- (Joris et al., 1993; Gilljam et al., 1989; Smith et al., 1996). In addition, in healthy individuals, ASF appears to contain the bactericidal factors known as defensin molecules (Zhang et al., 2001; Smith et al., 1996). In CF, however, it has been shown that these factors are lacking or less effective, leading to chronic airway infections (Smith et al., 1996). How these factors are inhibited in CF is somewhat controversial and currently there are two competing hypotheses.

The "compositional hypothesis" focuses on the role of CFTR as a Cl^- channel and its role in maintaining the normal electrolyte balance of ASF (by regulating electrolyte transport across the membrane) (Smith et al., 1996; Zabner et al., 1998). Antibacterial factors, normally contained within the ASF, are activated with low salt concentrations (Smith et al., 1996; Zabner et al., 1998). The malfunctioning CFTR and hence defective electrolyte transport will result in high salt concentration of ASF thus inactivating its bactericidal activity and leading to chronic infections (Smith et al., 1996; Zabner et al., 1998). As a side-effect, the buildup of neutrophils and macrophages in the airways in response to

infection also occurs, which leads to an inflammatory environment eventually causing hypersecretion of mucus and its build up (Smith et al., 1996).

On the other hand, the "low-volume hypothesis" focuses on CFTR as both a Cl^- channel and a regulator of ENaC and therefore CFTR affects both Na^+ and water transport (Mall et al., 2004). This hypothesis emphasizes the importance of proper ASF volume or depth to promote lung clearance by cilia and thus aid in clearance of mucus and infection. In CF patients, a decrease of Cl^- absorption at the surface epithelium leads to an increase in absorption of Na^+ and consequently water (Matsui et al., 1998; Knowles and Boucher, 2002; Mall et al., 2004). As a result the ASF volume is reduced, and according to the "low-volume hypotheses," this causes the build up of mucus. In healthy individuals, the ASF volume depth is slightly greater than the height of outstretched cilia, which aids in the free movement of cilia and therefore the mucus clearance out of the lungs (Mall, 2004). With the reduction of ASF volume and depth, cilia can not move freely and there is consequently a decreased mucus transport, and buildup of bacteria (Mall, 2004). The buildup of bacteria leads to chronic infections and recruitment of neutrophils, which can trigger goblet cell metaplasia and mucin hypersecretion (Mall, 2004).

Airway pathophysiology in CF is characterized by thick, sticky mucus on the surface epithelium. Due to thick mucus, clogging of bronchial passages also occurs, leading to difficulties in breathing. Thick mucus coupled with the lack of apparent ASF antibacterial function leads to reduced clearing of normal airway mucus and build up of bacteria. CF patients often sustain infections that

progressively destroy the normal functioning of lungs. About 90% of CF patients will eventually die due to lung disease infections (Welsh and Smith, 1995). The bacteria most often found to cause severe infections and/or are colonized at the time of death in the airways of CF patients are *Staphylococcus aureus* and *Pseduomonas aeruginosa* (Welsh and Smith, 1995). As opposed to the skin pathophysiology, lung disease is more difficult to explain solely due to a defect in Cl^- secretion.

2.3. CF Effect in the GI Tract

The liver and pancreas of CF patients also are characterized by plugging and obstruction of ducts, which results in impairments in digestion. While the liver is affected in only a rather small portion of patients (about 5%), the pancreas is one of the more severely affected organs (Welsh and Smith, 1995). CF frequently leads to a condition termed "pancreatic insufficiency" (Welsh and Smith, 1995). Only about 10% of CF patients will escape pancreatic insufficiency, this being due to different genotypes which result in milder forms of CF (Quinton, 1999). In healthy individuals, the acini in the pancreas secrete a fluid rich in enzymes and other proteins (Berne et al., 2004). The pancreatic duct cells are mainly responsible for secretion of a HCO_3^--rich fluid in response to hormones such as vasoactive intestinal peptide (VIP) and secretin (Ahn et al., 2001). Proteins secreted by acinar cells are kept inactive and in solution while flowing through the ducts by this copious secretion of alkaline fluid (Quinton, 1999; Lee et al., 1999a). In CF patients there is a decrease in Cl^- and HCO_3^- - dependent

fluid secretion and an increase in macromolecule concentration (i.e. protein) in pancreatic secretions (Quinton, 1999; Ahn et al., 2001). When there is a reduction of anion secretion, and thus water volume, the proteins become insoluble, precipitate and clog the smaller pancreatic ducts (Quinton, 1999; Ahn et al., 2001). In most patients (about 85%), the obstruction of the pancreatic ducts and resulting lack of digestive enzymes delivered to the duodenum results in pancreatic insufficiency and necessitates consumption of supplements providing additional digestive enzymes (Welsh and Smith, 1995). Loss of HCO_3^- homeostasis in the pancreas of CF patients requires a more complex explanation than solely a loss of CFTR and its function as a Cl^- channel.

The small intestines are only affected in a small percentage of newborns (about 10%) where they are obstructed by thick stool, a condition termed meconium ileus (Welsh and Smith, 1995). Prior to the 20th century, this condition was lethal. Now, however, it can often be relieved quickly by simple procedures, but sometimes does require surgery in newborns. Many CF patients also are at risk of developing deficiencies of fat-soluble vitamins (A, D, E and K) due to malabsorption, especially those individuals with pancreatic insufficiency and/or hepatobiliary disease (Rashid et al., 1999).

2.4. CF Effect in the Reproductive Tract

The reproductive tract also is often negatively affected in CF. In males, this is expressed through a condition called congenital bilateral absence of vas deferens (CBAVD) resulting in incomplete development of fine ducts (vas

deferens) (Timmreck et al., 2003). This condition, thought to be the result of blockage of ductules *in utero*, leads to sterility, and was only recently linked to mutations in CFTR (Quinton, 1999; Timmreck et al., 2003). In females the disease is exhibited through a prevalent thick mucus secretion obstructing the cervical canal and resulting, in many cases, in infertility.

3. Molecular Biology of CF

When expressed, the CFTR protein is localized in the plasma membrane of epithelial cells. CFTR belongs to the family of membrane proteins known as ATP-binding cassette (ABC) transporters (Riordan et al., 1989). ABC transporters are integral membrane proteins composed of two transmembrane domains, that in bacteria, utilize the energy of ATP to pump nutrients across the membrane (Jones and George, 2004). The amino acid sequences of the two transmembrane domains vary considerably (Jones and George, 2004). In addition, they also can be involved in the pumping of chemotherapeutic drugs from cancer cells, leading to development of drug-resistance (Jones and George, 2004). All ABC transporters contain a conserved ATP-binding cassette region, also known as the nucleotide binding domain (NBD), which is involved in ATP binding and hydrolysis (Walker et al., 1982). The NBDs of ABC transporters contain conserved Walker A (GXS/TGXGKS/T) and B (RX_{6-8}hhhhD) motifs as well as a C motif or signature sequence (LSXGXR/K), where the symbols "X" stands for any amino acid residue, and "h" for any hydrophobic residue (Zou and Hwang, 2001). Walker A and B motifs have been shown to be involved in ATP

binding (Saraste et al., 1990). The C motif is thought to participate in transfer of the energy of ATP hydrolysis to the conformational change leading to transport function (Ames and Lecar, 1992).

The human CFTR contains 1480 amino acid residues and has a molecular mass of ~168 kDa when fully glycosylated at two residues (Riordan et al., 1989). CFTRcontains several structural parts typical for the family of ABC transporter; two sets of six helical membrane-spanning domains (MSD1 and MSD2), a cytoplasmic regulatory (R) domain, and two cytoplasmic nucleotide (ATP) binding domains (NBD1 and NBD2) (Figure 1) (Sheppard and Welsh, 1999; Zou and Hwang, 2001). Despite conserved structural domains and three conserved sequences in NBDs, CFTR has very little similarities in function with other members of this superfamily. The opening of the channel and its function as primarily a Cl^- channel, appears to be allowed only upon phosphorylation of the R domain and cleavage of ATP by the nucleotide binding domains (Sheppard and Welsh, 1999). The two MSDs are believed to form the actual channel pore (Sheppard and Welsh, 1999). The R domain and its phosphorylation by PKA allow for the activated channel, while ATP hydrolysis by NBD domains appears to control channel gating and, hence its open cycle (Sheppard and Welsh, 1999). The unphosphorylated R domain seems to have an inhibitory effect on the channel (Rich et al., 1991). When removed experimentally, it results in a constitutively active channel (Rich et al., 1991). The channel will open only with rising levels of intracellular cAMP and subsequent phosphorylation of R domain

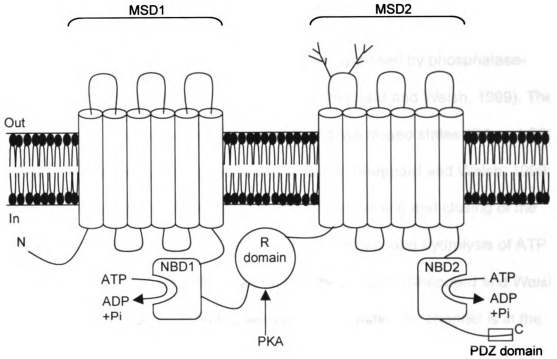

Figure 1. CFTR Structure. CFTR belongs to the family of membrane proteins referred to as ATP-binding cassette (ABC) transporters (integral membrane proteins composed of two transmembrane domains). The human CFTR contains 1480 amino acid residues. It contains several structural parts typical for ABC transporters, two sets of six helical membrane-spanning domains (MSD1 and MSD2), a cytoplasmic regulatory (R) domain, and two cytoplasmic nucleotide (ATP) binding domains (NBD1 and NBD2). The two MSD domains are believed to form the actual channel pore. The opening of the channel is allowed only upon a rise in cAMP levels and subsequent phosphorylation of the R domain by PKA at serine and threonine residues, and cleavage of ATP by the nucleotide binding domains. The R domain and its phosphorylation by PKA allows for the activated channel, while ATP hydrolysis by NBD domains appears to control channel gating and, hence its open cycle. Once the phosphorylation of the R domain occurs, the channel open cycle is controlled by hydrolysis of ATP by NBD domains and is closed by phosphatases' dephosphorylation of the R domain. The CFTR is said to have two open (O1 and O2) and two closed states (C1 and C2), defined by ATPs and/or ADPs bound to the NBD domains. At the C-terminal tail of CFTR (the very last three amino acids, Thr-Arg-Leu) are so called PDZ domain(s) (protein interaction domains). Proteins with PDZ domains can bind to other proteins with corresponding PDZ domains and physically influence each other's cellular functions, hence playing an important role in cellular signaling.

by PKA at serine and threonine residues (Zou and Hwang, 2001). Once the phosphorylation of the the R domain occurs, the channel open cycle is controlled by hydrolysis of ATP by NBD domains and is finally closed by phosphatase-mediated dephosphorylation of the R domain (Sheppard and Welsh, 1999). The CFTR is said to have two open (O1 and O2) and two closed states (C1 and C2), defined by ATPs and/or ADPs bound to the NBDs (Sheppard and Welsh, 1999; Zou and Hwang, 2001). The simplest model of the opening and closing of the CFTR is that upon the phosphorylation of the R domain, and hydrolysis of ATP on one of the NBD domains, the channel is in its O1 state (Sheppard and Welsh, 1999). Upon ATP hydrolysis on the second NBD domain, the channel is in the O2 state and will soon be closed. Two closed states are defined at the release of two ADPs and binding of the first ATP molecule to the NBD1 domain (C1) followed by consequent binding of a second ATP molecule to the NBD2 domain and phosphorylation of the R domain (C2) (Sheppard and Welsh, 1999). Various models have been proposed to explain timing and order of these states such as phosphorylation of the R domain, release of ADP, binding of ATP and its hydrolysis, and crosstalk between the domains. The exact details of gating and conformational states, however, are still unknown.

Another important structural feature of CFTR is the so called PDZ domain located at the C terminal tail of CFTR (Figure 1) (Raghuram et al., 2003; Haggie et al., 2004). The very last three amino acids of the C-terminus of CFTR (Thr-Arg-Leu) match the conserved PDZ motif found in many PDZ domain-containing proteins (Haggie et al., 2004). PDZ domains are protein interaction domains

located on many eukaryotic proteins (Sheng and Sala, 2001). Protein-protein interaction through PDZ domains is based on sequence specificity. They are used for intracellular trafficking of proteins and subcellular localization of protein complexes and hence, play an important role in cellular signaling (Sheng and Sala, 2001). Proteins with PDZ domains can bind to other proteins with corresponding PDZ domains and physically influence each other's cellular functions. CFTR has been shown to influence (both directly and indirectly) the function of other membrane transport proteins such as epithelial Na^+ channels (ENaC), outwardly rectifying Cl^- channels, Cl^-/HCO_3^- exchangers, renal K^+ channels (ROMK), aquaporin type III water channels (AQP3), Na^+/H^+ exchanger isoform 3 (NHE3), and Ca^{2+} activated Cl- channels, (Kunzelmann and Schreiber, 1999; Schwiebert et al., 1999). This influence has been suggested to be mediated through PDZ domain protein-protein interactions (Kunzelmann and Schreiber, 1999). It also has been shown that CFTR is confined to the apical membrane of epithelial cells and that its activity can be modulated through the PDZ domain interactions with the above mentioned complexes (Raghuram et al., 2003). The C-terminal tail of CFTR has been shown to interact with, and be regulated by, PDZ domains of Na^+/H^+ exchanger regulatory factors (NHERF) and CAP70 (Wang et al., 2000; Raghuram et al., 2001).

Malfunction of CFTR can be caused by numerous mutations in any of the above-mentioned domains, leading to various degrees of disease severity. So far, more than 1,000 mutations have been identified in CF that could lead to various defects in CFTR biosynthesis, its function on the membrane, and its

stability (Amaral, 2004). The most common mutation, deletion of phenylalanine at position 508 (ΔF508), accounts for about 70% of all cases of CF (Cheng et al., 1990). This mutation is located within the first nucleotide binding domain (Cheng et al., 1990). Cheng and colleagues were one of the first groups to report what occurs as a result of this particular mutation. They showed that the ΔF508 mutation leads to a large decrease of fully glycosylated CFTR in cells (Cheng et al., 1990). They concluded that this mutation results in the protein being retained within the endoplasmic reticulum (ER) due to incomplete processing that eventually leads to protein degradation. The effect of this mutation is "incomplete processing" by the endoplasmic reticulum early in the translation process (prior to translation of the ATP binding site) and a consequent lack of trafficking to the membrane. Sato et al. (1996) later showed that this defect does not result in a dysfunctional protein if it is successfully processed to the membrane. They demonstrated the release of ΔF508 CFTR protein from the ER and its correct processing to the plasma membrane when cells were treated with 10% glycerol. The protein was shown to be partially functional with the expected increase of cAMP-activated chloride current. In fact, recent findings indicate that more than 50% of the wild type (WT) CFTR translated becomes a misfolded protein and is retained within the ER and subsequently degraded (Sharma et al., 2001). The remainder of the WT protein (25-50%) will be released from ER and will undergo further glycosylation and processing in the Golgi apparatus and finally be exported to the cell membrane (Sharma et al., 2001). Glycerol treatment of cells was shown to stabilize the mature protein, and not only corrects trafficking of

ΔF508 CFTR, but also facilitates the processing of the WT (Sato et al., 1996). The stabilization defect of ΔF508 mutation was also shown to be a temperature-sensitive defect (Sharma et al., 2001). The introduction of other chemical chaperones, reduced temperature, and down-regulation of Hsp70 (a molecular chaperone) were found to partially correct the effects of the ΔF508 mutation (Sharma et al., 2001). Specifically, misfolded ΔF508 CFTR does not process properly, most likely due to association with molecular chaperones such as Hsp70/Hdj-1 and calnexin, and as a result enters the ubiquitin-proteosome degradation pathway (Pind et al., 1994; Amaral, 2004).

4. Contemporary Views on CFTR

The first direct demonstration of CF as a 'Cl^- transport defect' was reported in 1983 (Quinton, 1983). This study reported that in isolated sweat ducts low Cl^- permeability in ducts resulted in poor reabsorption of NaCl and high NaCl concentration in the sweat of CF patients. Most of the research done in the field of cystic fibrosis so far has been focused on this interrupted chloride transport of CFTR. It has been shown that many CF mutations do cause this defective Cl^- transport (Ahn et al., 2001). It also has been shown, however, that high or normal Cl^- transport is maintained in several CFTR mutations which still cause mild and in some cases severe forms of CF (Ahn et al., 2001). This suggests that CFTR most likely has more roles than previously proposed by contemporary evidence of a Cl^- transport defect. Since the first reports indicating a Cl^- transport defect as the underlying problem of CF, CFTR has been found to be a global regulator of

epithelial fluid and electrolyte transport influencing and/or regulating various functions including 1) Cl^- secretion through Cl^- channels, 2) Na^+ absorption through ENaC, 3) K^+ secretion through luminal K^+ channels, and 4) HCO_3^- secretion through Cl^-/HCO_3^- exchangers, and 5) HCO_3^- salvage through Na^+/H^- exchangers (Lee et al., 1999b; Ahn et al., 2001). The influence and role of CFTR on the many facets of epithelial fluid and electrolyte transport has been a focus of much recent research.

5. Shmuel Muallem's Work

Recently, groups from the University of Texas Southwestern and Yonsei University in Seoul, Korea, headed by Shmuel Muallem published new findings that have made dramatic impact in the field of cystic fibrosis (Choi et al., 2001a; Choi et al., 2001b). Muallem's group was interested in impaired HCO_3^- secretion in all CFTR expressing tissues and in CF in general, which was, and still is, poorly understood. HCO_3^- rich fluid secretion in these tissues is normally high (containing 100 – 140 mM of HCO_3^-) and is important for maintenance of protein stability and/or their inactive state (for example, digestive enzymes in pancreas and mucins in other tissues) (Lee et al., 1999a). Previously it was hypothesized that HCO_3^- secretion in CFTR-expressing tissues is mediated through electrogenic as well as electroneutral pathways (Lee et al., 1999a). An electrogenic pathway could involve either an unknown HCO_3^- channel or CFTR itself (Illek et al., 1997; Seidler et al., 1997). Electroneutral secretion most likely would involve a Cl^-/HCO_3^- anion exchanger (AE) (Lee et al., 1999a).

In their 1999 studies, Lee and colleagues examined the regulation of HCO_3^- secretion by CFTR. In their first study, they used HEK 293 and NIH 3T3 cell lines expressing CFTR. From patch clamp and internal pH studies, they concluded that the major pathway for HCO_3^- secretion in these cells was through an AE that was directly regulated by CFTR (Lee et al., 1999a). They found that secretion was dependent on membrane expression and cAMP-activated conformation of CFTR, independent of CFTR function as a Cl^- conductor, and inhibited by mutations in NBD2 of CFTR.

Later that year, they published another set of studies in which they evaluated the physiological importance of their previous findings(Lee et al., 1999b). CFTR regulation of Cl^-/HCO_3^- exchange was examined in cells naturally expressing high levels of CFTR in the luminal membrane (the human colonic T84 cell line, mouse submandibular glands [SMG] and pancreatic ducts) (Lee et al., 1999b). Similar findings as previously were recorded where: 1) stimulation with forskolin (a CFTR stimulator) exerted positive effects on AE in T84 cells independent of Cl^- conductance through CFTR, and 2) in studies using SMG and pancreatic ducts isolated from transgenic mice, CFTR was found to regulate luminal AE activity but not basolateral AE's. The luminal AE in question was not identified, but they speculated that it might be AE3 or a yet unidentified isoform. HCO_3^- homeostasis (impaired in many CFTR expressing tissues) is sustained through both HCO_3^- secretion and salvage mechanisms (Ahn et al., 2001).

Concurrently with their studies examining CFTR control of HCO_3^- secretion, this group also was interested in HCO_3^- salvage mechanisms. In their

2001 study, Ahn and colleagues studied mouse pancreatic ducts and heterologous expression systems and hypothesized that CFTR also was involved in the control of these salvage mechanisms, specifically by influencing the activity of the Na^+/H^+ exchanger (NHE3) (Ahn et al., 2001). They found that CFTR control of NHE3 activity was mediated through two mechanisms: 1) by increasing cAMP dependent inhibition of NHE3, and 2) by increasing expression of NHE3 in luminal membranes of pancreatic duct cells (Ahn et al., 2001). The molecular mechanism of this control, they hypothesized, was signaled through PDZ interaction and binding of CFTR and NHE3 to one of the PDZ scaffolding proteins. This also was further supported by CFTR and NHE3 co-immunoprecipitation in PS120 cells and observation that the interaction was dependent on the C-terminal PDZ motif of CFTR (Ahn et al., 2001)

In more recent work (Choi et al., 2001a) this group studied HEK293 cells transiently transfected to express various pancreatic sufficient and insufficient mutations (mutations that result in a functional pancreas and those resulting in complete pancreatic dysfunction respectively) of CF. Pancreatic sufficient mutations used were as follows: E193K, G551S, D648V, A800G, H949Y and R1070Q; pancreatic insufficient mutations used were: I148T, G178R, R297Q, G551D, H620Q, G970R, A1067T, G1244E, S1255P, and G1349D (Choi et al., 2001a). Surprisingly, they showed that some pancreatic insufficient mutations had normal Cl^- transport while HCO_3^- transport and pH regulation were aberrant (Choi et al., 2001a). These results showed that HCO_3^- transport and pH control might have an equal if not greater role than chloride transport in the pancreatic

pathophysiology of CF. In a follow up paper (Choi et al., 2001b), the authors hypothesized that CFTR may also be a Cl^-/ HCO_3^- exchanger. These findings renewed interest in alternative defects linked to CFTR malfunction, especially dysregulation of HCO_3^- conductance and extracellular pH (pH_o).

6. Working Hypothesis in This Study

The primary research aims of our laboratory are to test the human cystic fibrosis transmembrane conductance regulator (CFTR) for the capacity to regulate extracellular pH. As a result of previous reports in the literature and findings in our laboratory, our working hypothesis is that the extracellular pH of the milieu surrounding CF cells (CFTR- "minus") is more acidic than that of normal cells (CFTR+ "plus") and that under optimum conditions a very sensitive pH probe can differentiate CF from normal cells. This study, in particular, aimed to characterize the bicarbonate transport function of CFTR. We designed an experimental approach that enabled us to closely examine the capacity of CFTR to carry bicarbonate and chloride, and to test CFTR as a possible Cl^-/ HCO_3^- exchanger.

In our data collection, we utilized microphysiometer technology to determine the importance of pH and HCO_3^- in CF. We utilized a pH biosensor to detect extracellular pH changes around CF cells in culture. This project was made feasible by a technology in the form of a silicon chip-biosensor, the microphysiometer (Molecular Devices Corporation, Sunnyvale CA), that can detect subtle changes in extracellular pH (McConnell et al., 1992). The

microphysiometer, a semiconducter-based instrument, detects the rate at which a living cell acidifies its extracellular environment. The device can detect changes in extracellular pH as a result of transient acid/base fluxes such as those caused by short-lived transporter activity, alterations in metabolic rate, and changes in intracellular pH (McConnell et al., 1992). We also used an ORION iodide electrode system to measure and directly record anion flux through the CFTR channel expressed in mammalian cell lines.

In early 2001, our laboratory published findings using CFTR-expressing (2WT2 and 3T3WT) and non-expressing cell lines (C127, NIH/3T3) that indicated that CFTR-expressing cells alkalinize extracellular media in response to forskolin while CFTR-deficient cells (as is the case in the disease) acidify it. This could be the result of either increased base efflux or decreased acid efflux in CFTR-expressing cells (Luckie and Wine, 1998; Luckie et al., 2001). Results from our studies and from the literature suggest that the CFTR may normally be involved in significant HCO_3^- conductance, and that in the disease state, pH regulation is disrupted resulting in the acidification of the environment of the CF lung (Smith and Welsh, 1992; Poulsen et al., 1994; Luckie et al., 2001; Coakley et al., 2001; Tate et al., 2002; Coakley et al., 2003). Interestingly, changes in extracellular pH (pH_o) in pancreatic secretions lead to premature activation of zymogens while changes of pH_o of the surface fluid in the CF lung may contribute to the inactivation of natural immune defenses that occurs in CF (Smith et al., 1996). Hence, an understanding of CFTR HCO_3^- conductance may prove critical to

understanding CF lung and pancreatic disease, the lethal components of cystic fibrosis.

7. Specific Aims of the Research

The purpose of this study was to characterize bicarbonate conductance through CFTR and to examine the "Muallem hypothesis" that CFTR is an anion exchanger (AE). The specific aims of my research were; 1) to characterize the Cl^- channel conductance properties of CFTR in selected cell lines, 2) to examine the conditions necessary for CFTR-dependent HCO_3^- conductance, and 3) to test the Muallem hypothesis. My study focused on two cell lines, 2WT2 and ΔF508-8. Both cell lines are derived from C127 mouse mammary epithelial cells. They are stable cell lines transfected with the BPV vector carrying wild type (2WT2) and mutant CFTR cDNA (ΔF508-8) (Marshall et al., 1994).

Direct characterization of the Cl^- channel conductance properties of CFTR in our cell lines was achieved through iodide efflux experiments. We used an ORION iodide electrode to measure and record iodide (anion) flux through CFTR in transfected cells. Iodide efflux-characterized Cl^- channel conductance properties and activity was assessed by monitoring the responses to inhibitors (glibenclamide, DPC and DIDS) and activators of this channel (forskolin). We assumed that the majority of iodide efflux observed was mediated by Cl^- channels (mainly CFTR). AE mechanisms prefer Cl^- over I^-, theoretically by a factor of ~260, thus the transport of I^- through such mechanisms would be insignificant (Venglarik et al., 1990). Experimentally researchers have shown that

the I^- does not compete with Cl^- with AE mechanisms, further supporting this assumption (Humphreys et al., 1994). Iodide efflux was expected to be observed in both cell lines but it was also expected to be much higher in wild type cells in response to forskolin (CFTR stimulator).

Examination of the conditions necessary for CFTR dependent HCO_3^- conductance were achieved through the use of microphysiometry. The microphysiometer, a semiconducter-based instrument, detects the rate at which a cell acidifies its extracellular environment. HCO_3^- is the cells' most important physiological pH buffer, and thus, by detecting extracellular pH changes, we were able to interpret HCO_3^- movement through CFTR. Cells were perfused with physiological buffers containing various CFTR and AE activators and inhibitors (as listed above) and resulting changes in pH_o were dynamically monitored.

Finally, examination of the Muallem hypothesis (the third aim of my research), was achieved through the above mentioned cytosensor and iodide studies as well as by extensive literature research and review. In evaluating Muallem's hypothesis we assumed that CFTR channel and exchanger properties, as proposed by this group, are two separate entities. We focused on three predictions proposed by Muallem's group; 1) bicarbonate transport should be Cl^- dependent and stimulated by forskolin, 2) glibenclamide (a known CFTR inhibitor) should stop efflux of chloride through the channel but should not prevent the action of CFTR as an exchanger, and 3) DIDS (an anion exchanger blocker) should affect and block bicarbonate movement thorough CFTR's anion exchanger in cells expressing CFTR.

MATERIALS AND METHODS

1. Cell Lines

In the current studies of iodide efflux and extracellular acidification, we used three different cell lines of C127 (also referred to as C127i) mouse mammary epithelial origin. The lines were developed by Dr. Seng Cheng, supplied by Genzyme Corporation, and have been well characterized (Denning et al., 1992).

These cell lines are C127 parental cells stably transfected by the calcium phosphate precipitation method. The cells were transfected with bovine papilloma-based vector (BPV) alone, or containing the cDNA of wild type or ΔF508 mutated CFTR (pBPV-CFTR and pBPV-ΔF508 respectively) under the control of the mouse metallothionein MT1 promoter (Figure 2) (Marshall et al., 1994). Expression vectors were made by inserting a fragment from the high copy number plasmid pMT-CFTR4 containing the entire human CFTR cDNA into eukaryotic expression vector CLH3AXBPVXT-NEO (Reddy et al., 1987; Cheng et al., 1990; Marshall et al., 1994). ΔF508 mutant CFTR was previously constructed by oligonucleotide-directed mutagenesis into the pMT vector and was then transferred to CLH3AXBPVXT-NEO using the same procedures as in the case of wild type CFTR (Cheng et al., 1990; Marshall et al., 1994). The pBPV vectors also contained the BPV genome, neomycin resistance gene under the control of another copy of the MT1 promoter, pML (derivative of pBR322), and a 1.9-kb intron of the α- subunit of human fertility hormones (Figure 2) (Marshall et al.,

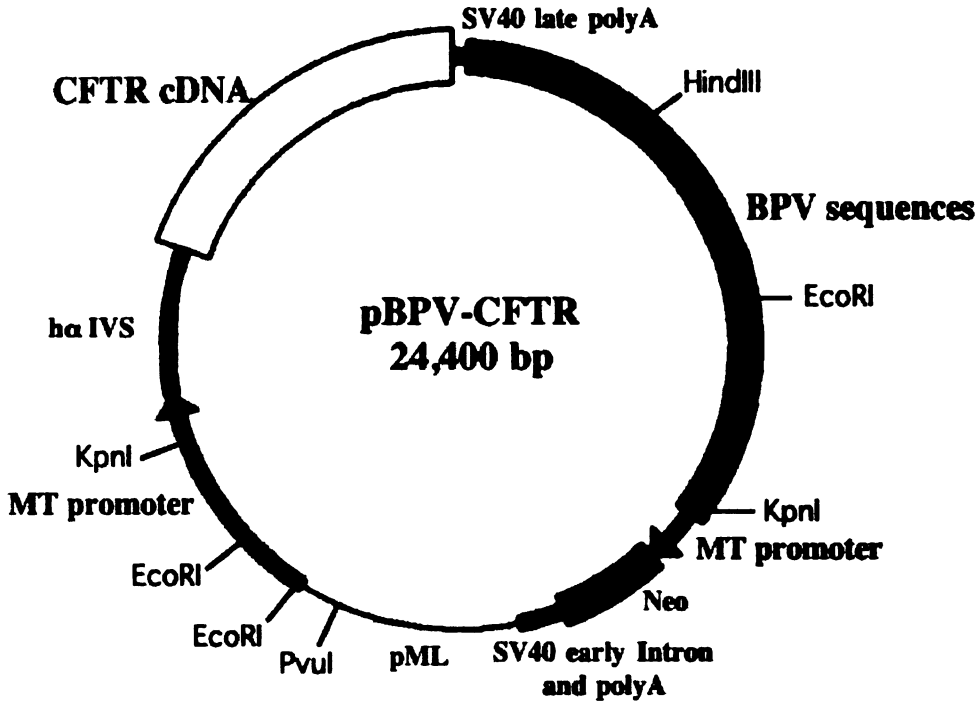

Figure 2. Mammalian expression vector pBPV-CFTR. The pBPV vectors were used to transfect C127 mouse mammary epithelial cells to express wild type or mutated ΔF508 CFTR (Marshall, et al., 1994). The vector was composed of human CFTR cDNA under the control of mouse metallothionein MT1 promoter obtained from high copy number plasmid pMT-CFTR4 (Cheng, et al., 1990). It also contained BPV genome to promote self-replication and maintenance of plasmid in C127 cells, neomycin resistance gene under the control of another copy of MT1 promoter for selection purposes in mammalian cells, pML (derivative of pBR322) for propagation and selection of plasmid in *Escherichia coli*, and 1.9-kb intron of the α-subunit of human fertility hormones shown that when placed at 5' untranslated region it increases efficiency of RNA processing and cytoplasmic accumulation (Marshall et al., 1994). The C127 cells were transfected by the calcium phosphate precipitation method and cells were selected for by G418 treatment. ΔF508 mutant CFTR was previously constructed by oligonucleotide-directed mutagenesis into pMT vector and was then transferred to CLH3AXBPVXT-NEO using the same procedures as in the case of wild type CFTR thus obtaining pBPV-ΔF508 (Cheng et al., 1990; Marshall et al., 1994).

1994). Transfected C127 cells used in this study are denoted as BPV cells (transfected with BPV vector alone), 2WT2 cells (transfected with BPV vector containing wild type CFTR cDNA) or 508-8 cells (transfected with BPV vector containing cDNA of CFTR containing deletion of three bases resulting in absence of phenylalanine at position 508). In the current work, they will are referred to as wild type (2WT2 cells), mutant (508-8 cells) and control (BPV cells).

The pBPV-CFTR and pBPV-ΔF508 transfected C127 cells were shown to produce high levels of recombinant human CFTR (in case of wild type cells pretreated with butyrate, the limit of production was up to 1-5 x 10^5 mature CFTR molecules/cell) (Marshall et al., 1994). The processing of mature, glycosylated CFTR, however, has been shown to be ineffective. Only about 40% of produced wild type CFTR was converted to the mature form and expressed as functional Cl^- conducting channel at the plasma membrane (Marshall et al., 1994). The rest of the produced protein was rapidly degraded in a pre-Golgi compartment. Similarly, ΔF508 mutated CFTR in 508-8 cells was found to be arrested in the endoplasmic reticulum and not detected at the plasma membrane (Marshall et al., 1994).

2. Cell Culture

Cell lines were stored in liquid nitrogen until needed. They were thawed over the course of 5 min in the standard Dulbecco's Modified Eagle Medium (DMEM). When cultured, they were only used up to passage number 43 (for wild type cells), 37 (for mutant cells) and 21 (for control cells). Thawed cells were

maintained in an incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS), 5% penicillin-streptomycin cocktail (P/S), prepared with 10,000 units/ml penicillin G sodium and 10,000 μg/ml streptomycin sulfate in 0.85% saline, and 0.2 mg/ml G418. The medium was changed every 2-3 days. Cell growth was monitored and when 95-100% confluency was reached, cells were passaged after detachment with a Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline wash for 5 min at 37°C and by 10 min of 0.25% trypsin, 1 mM EDTA treatment at 37°C. Detached cells were passaged to either a desired flask (for further culturing), 35 mm cell culture dishes (for iodide efflux experiments) or 12 well plate inserts (for microphysiometer studies).

3. Iodide Efflux

The presence of functional CFTR was assayed by cAMP-dependent activation of Cl^- channels in iodide efflux experiments. We used an ORION® iodide electrode to measure and record iodide (anion) flux through CFTR in transfected cells. The C127 cells (wild type, mutant and control) were maintained in T25 flasks in a 37°C incubator in standard DMEM supplemented with 10% FBS and 5% P/S. When confluency of 100% was reached, they were trypsinized and passaged into five, 35 mm sterile polystyrene cell culture dishes and grown in the same conditions until 95-100% confluency was reached. At this point cells were treated with 0.2 mg/ml G418 and left in the incubator over night. On the day of the experiment, the cells were first incubated in 1.0 ml of 37°C efflux buffer

(5.4 mM KCl, 0.8 mM $MgSO_4$, 10.0 mM HEPES, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 150.0 mM NaCl and 1.0 mg/ml glucose) for 20 min. Efflux buffer was then removed and dishes were filled with 1.0 ml of loading buffer (5.4 mM KCl, 0.8 mM $MgSO_4$, 10.0 mM HEPES, 1.0 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 150.0 mM NaI and 1.0 mg/ml glucose). The cells were then placed in a 37°C incubator (in a humidified atmosphere of 95% air and 5% CO_2) for an hour to allow equilibration of sodium iodide inside and out of cells.

Following the loading period, iodide efflux through CFTR was measured using the complete sample replacement technique of Venglarik et al. (1990). Drug solutions prepared in efflux buffer (EB) were: 1) 10 μM forskolin, 2) 400 μM glibenclamide + 100 μM Diphenylamine-2-dicarboxylate (DPC) + 10 μM forskolin, 3) 500 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) + 10 μM forskolin, and as vehicle control for glibenclamide treatments 4) dimethyl sulfoxide (DMSO) + 10 μM forskolin (Table 1). Stock glibenclamide was diluted in DMSO and when added to EB the final DMSO concentration equaled 0.4%. For each 35 mm culture dish of cells, there were 12 measuring points including the collection of the remaining loading buffer through cell lysis at the end of the experiment. The first collection vial (wash) included the loading buffer and four washes with 1 ml EB. Following the wash, 1 ml of EB was placed on each cell dish for 30 seconds and the efflux fluid was collected (vials 2-6). Vials 7-11 were collected in the same way, however, they included 30 sec with 1 ml of EB with appropriate drug treatment (for experimental vials) and control. For the last treatment (at the five minute point), lysis buffer (2N NaOH), was used in order to

Table 1. Drug treatments and/or buffers used in iodide efflux and microphysiometer studies. The following drug treatments and/or solutions were used in studies as designated in order to characterize CFTR as channel or exchanger. Not all treatments were used in both set of studies. At times treatments were combined and used simultaneously as denoted in method section and figures representing the results.

Drug treatments and/or buffers	Function	Iodide Efflux	Microphysiometry
10 μM forskolin	CFTR agonist	Applied	Applied
400 μM glybenclamide + 100 μM DPC	CFTR antagonist	Applied	Applied
500 μM DIDS	Anion exchanger antagonist	Applied	Applied
0.4% DMSO	Vehicle control for 400 mM glybenclamide treatment	Applied	Not used
Cl^- free buffer	To examine CFTR as a Cl^-/HCO_3^- exchanger	Not used	Applied
Loading buffer (with iodide)	To load cells with iodide the efflux rate of which will then be observed	Applied	Not used
Efflux buffer (no iodide)	To create concentration gradient for iodide to exit cells	Applied	Not used

determine the amount of sodium iodide that remained in the cells. All of the previous time points could then be evaluated relative to the iodide lysis count.

Iodide measurements for each vial were recorded in the next 24-48 hr using the ORION® iodide electrode (Model 9653 **ion**plus™ Series) following the instructions provided by the manufacturer. Before each 'reading session,' the iodide electrode was calibrated with NaI standards (10 μM, 100 μM, 1 mM, 10 mM, and 100 mM), and an ionic strength adjuster (5.0 M $NaNO_3$) was added to all samples per manufacturer's instructions to adjust to a constant background ionic strength. Data are presented as rate constant of efflux flow at each data collection point graphed as iodide efflux rate vs. time. Rate is defined as change in iodide concentration over time. We used the following equation to calculate the rate constant: $r = [\ln(R_1) - \ln(R_2)]/(t_1-t_2)$ where R_1 and R_2 represent the percent of iodide concentration remaining in the cell layer at times 1 (t_1) and 2 (t_2) respectively, as compared to iodide concentration in the lysis vial (Venglarik et al., 1990). When discussing the iodide efflux results, data are described as change in rate (iodide concentration) over time for the three different cell lines (wild type, mutant and control).

4. Measurement of Extracellular Acidification (Cytosensor Microphysiometry)

4.1. General Operating Principles

The microphysiometer, a semiconducter-based instrument, detects the rate at which a cell acidifies its extracellular environment. The device can detect

changes in extracellular pH (pH_o) as a result of transient acid/base fluxes (McConnell, et al., 1992). Cells are in diffusive contact with a semiconductor-based pH sensor, the light-addressable potentiometric sensor (LAPS) (Hafeman, et al., 1998; Parce et al., 1989) (Figure 3). The LAPS determines the pH in a Nernstian fashion (61 mV per pH unit change at 37°C) from changes in voltage and the voltage at the surface of the chip relative to acidification (McConnell et al., 1992).

Two fluid paths are associated with each cell chamber (four total), and both data recording and fluid path selection are computer controlled (Figure 4). In this study, cell chambers were used in which adherent epithelial C127 cells were grown on porous polycarbonate filters and during the assay retained between this and a second microporous polycarbonate membrane (Figure 3). Extracellular acidification rates were determined as the rate of change of sensor output during periodic interruptions of fluid flow that causes transient acidifications of < 0.1 pH unit. Acidification rates were calculated using a least-squares regression of data obtained during periodic interruptions of fluid flow (40 sec) and were reported in microvolts per second (μV/sec). One microvolt per second (μV/sec) corresponds closely to an acidification rate of 0.001 pH unit/min (at pH 7.4).

Since acidification rate varies with absolute pH, we always started the experiment at pH 7.4 and the total pH never varied by more the 0.1 pH unit for the duration of the experiment. When rates of acidification were compared across different chambers in which the absolute number of cells (and thus basal acidification rate) varied, the data were normalized to account for variation in

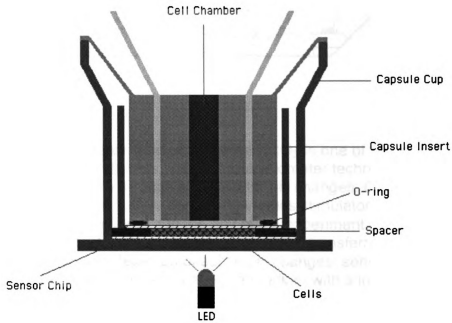

Figure 3. Assembled microflow (sensor) cell chamber. The cells were assembled in a microflow chamber (a 1.4 μl cylindrical space of 50 μm height and 6 mm diameter) in which they are in diffusive contact with a semiconductor-based pH sensor, the light-addressable potentiometric sensor (LAPS) (Hafeman, et al., 1988; Parce et al., 1989). The LAPS determines and records the extracellular acidification rates as the rate of change of sensor output during periodic interruptions of fluid flow. A two mm diameter circular region in the center of the cell capsule is used for pH recordings by a light emitting diode (LED) located underneath the sensor chamber. Cell chambers were used to grow adherent epithelial C127 cells on porous cell capsules. During the assay cells were retained between two microporous polycarbonate membranes (capsule cup and capsule insert). The cells and sensor chambers were first incubated with the running media. The spacer was then placed on the capsule cell surface and the capsule insert was placed on top of the spacer. Once the cell assemble was complete, the cell capsules were transferred to equilibrated sensor chambers with a silicon chip (pH detector) on the bottom and placed on to the recording instrument. The cell capsule was held to the bottom of the sensor chamber by a plunger and a gantry. The plunger was used to deliver continuous fluid flow to the cell layer using microflow cylinders that perfuse cells, bringing the media from a specified source and removing it to the waste container.

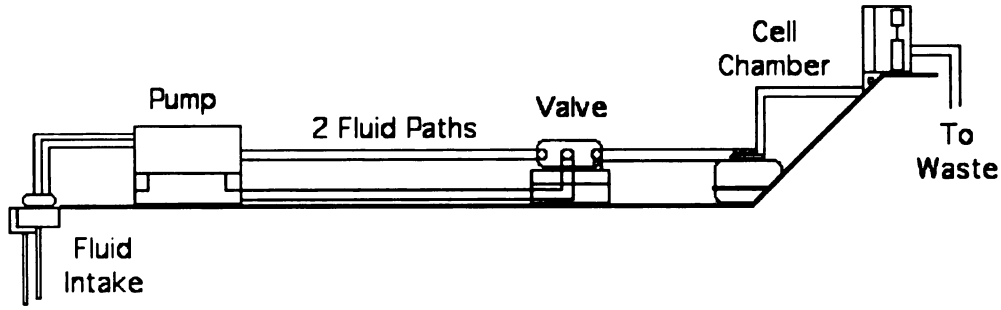

Figure 4. Microphysiometer fluid path. Since HCO_3^- is one of the most important physiological pH buffers, with microphysiometer technology we were able to detect HCO_3^- activity through extracellular pH changes. Cells were perfused with physiological buffers containing various stimulators and inhibitors and resulting pH_o was monitored. On the day of the experiment the cells were prepared as per manufacturer's recommendations and transferred into sensor chambers. To enhance the detection of subtle pH changes, sensor chambers were perfused with nominally bicarbonate-free medium with a low buffering capacity. Media was also degassed/debubbled and warmed to 37°C before perfusing the cells. Adherent cells were not manipulated in any way during an experiment, however pH_o was continually monitored. Two fluid paths are associated with each cell sensor chamber (four total), and both data recordings and fluid path selection is computer controlled. Reservoirs of media were connected to the flow chamber by tubing in which the flow was set to 100 μl/min. Directing flow from a given reservoir was done by software driven valves, one path perfusing the cells, and the other one directly being removed to the waste. Each recording cycle lasted two min and consisted of a perfusion phase and an interruption phase. During the perfusion phase, the media was continuously passed through the chamber for an interval of 80 sec. During the next phase (interruption phase), the pumps were inactivated for 40 sec, during which the rate of acidification within the chamber was calculated, recorded and plotted by the software (rate data). The flow was then resumed and the next cycle begun, with rates of acidification returning again to baseline. Once the rate of pH_o was constant drug treatments followed.

acidification levels between chambers. Normalization was performed by Molecular Devices' cytosensor software "Cytosoft 2.0.1." For normalization,steady state acidification rates of about 10 min prior to drug application were established as baseline and rate values (μV/s) were converted to percentage (%) change.

Since HCO_3^- is one of the most important physiological pH buffers, with microphysiometer technology we were able to study HCO_3^- flux through extracellular pH changes. Cells were perfused with physiological buffers containing various stimulators and inhibitors and resulting pH_o was monitored. Buffers used were nominally bicarbonate free with a low buffering capacity (2.0 mM HEPES and ~100 μM HCO_3^- from atmospheric CO_2), with the assumption that intracellular cell production of HCO_3^- should create a large driving force for HCO_3^- efflux (in our HCO_3^- free extracellular buffer) (Luckie, et al., 2001).

4.2. Specific Experimental Procedures

The C127 cells (control, wild type or mutant) were cultured in T25 flasks at 37°C incubator in standard DMEM supplemented with 10% FBS and 5% P/S (as described above). When confluency of 100% was reached, they were trypsinized with 0.6 ml of 0.25% trypsin, 1 mM EDTA solution, followed by a 1.0 ml DMEM wash and finally 0.2 ml of the resulting cell mixture was passaged into six 12-mm diameter disposable 3.0 mm porous polycarbonate cell capsules (Corning Incorporated). The cells were grown in the same conditions until 95-100% confluency was reached (about 2-3 days). Once confluency of 95-100% was

reached, they were treated with 0.2 mg/ml G418 and left in the incubator overnight. On the day of the experiment, the cells were prepared as per manufacturer's recommendations before transfer into sensor chambers (Figure 3). The cells and sensor chambers were first equilibrated with the running medium (148 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 2.0 mM HEPES, 1.8 mg/ml glucose; pH 7.4) for about 10 min. A spacer, which defines the internal size of the cell area used for recording, was then placed on the capsule cell surface (Figure 3). A capsule insert, which traps the cells between a pair of miroporous membranes (capsule and insert) and a spacer, was then placed on top (Figure 3).

Once the cell assembly was completed, the cell capsules were transferred to equilibrated sensor chambers with a silicon chip (pH detector) on the bottom and placed on to the recording instrument. The cell capsule was held to the bottom of sensor chamber by a plunger and gantry. The plunger also was used to deliver continuous fluid flow to the cell layer (Figure 3). A two mm diameter circular region in the center of cell capsule was used for pH recordings by a light emitting diode (LED) located underneath the sensor chamber (Figure 3). To enhance the detection of subtle pH changes, sensor chambers were perfused with a nominally bicarbonate-free running media with a low buffering capacity. Although bicarbonate is not added, atmospheric CO_2 will contribute to the solution. Concentration of bicarbonate can be estimated given a solubility of 0.592 ml CO_2/ml water and assuming room air is 740mm Hg with 0.0314% CO_2 (Brookes and Turner, 1994). The media also was degassed/debubbled and

warmed to 37°C before perfusing the cells. Adherent cells were not physically manipulated in any way during the experiment, but pH_o was continually monitored. The perfusion media also contained drugs where indicated. Reservoirs of cell media were connected to the flow chamber by tubing in which the flow rate was set to 100 μl/min (Figure 4). Directing flow from a given reservoir was done by a software driven valve system.

Each cycle lasted two min and consisted of a perfusion phase and an interruption phase. During the perfusion phase the medium was continuously passed through the chamber for an interval of 80 sec. During the interruption phase, the pumps were halted for 40 sec, during which the rate of acidification within the chamber was calculated, recorded and plotted by the software (termed rate data). The flow was then resumed and the next cycle begun, with rates of acidification returning again to baseline.

The standard protocol during an experiment is to allow all cells to stabilize prior testing. Once the rate of acidification stabilized and was constant for 10-20 min, drug treatments followed. Drug treatments used in the microphysiometer experiments were as follows: 1) 10 μM forskolin (10 min), 2) 400 μM glibenclamide + 100 μM DPC (10 min), 3) 400 μM glibenclamide + 100 μM DPC + 10 μM forskolin (10 min), 4) 500 μM DIDS (10 min), 5) 500 μM DIDS+ 10 μM forskolin (10 min), 6) Cl^- free buffer (148 mM Na gluconate, 5.0 mM K gluconate, 1.0 mM Mg gluconate, 1.0 mM Ca gluconate, and 2.0 mM HEPES, 1.8 mg/ml glucose; pH 7.4) (28 min followed by 10 min Cl^- free buffer + 10 μM forskolin and finished by another 12 min of Cl^- free) (Table 1). All drugs were dissolved in

running media unless otherwise specified. Each time perfusing media was changed, the cells were allowed to equilibrate until rate measurements become constant. Following completion of an experiment, rate data was normalized (10 min prior to drug treatment of interest) and data was exported to spreadsheet software Microsoft Excel, 2001 (Microsoft Corporation). The data was plotted as normalized acidification rate vs. time, and any differences in the normalized acidification rates in various cells (control, mutant and wild type) were determined.

5. Statistical Analysis

Except where noted, all data are reported as mean ± SEM. Statistical significance was assessed by performing two-tailed Student's t-tests as applied in Microsoft Excel. When dealing with microphysiometer data, the notation "n=x(y)" was used, where "x" stands for traditional trial number and denotes the number of cell capsules used in a single experiment. The "y" stands for number of multiple replications performed on a single cell capsule for the duration of a single experiment. For example n=2(4) would indicate two different cell capsules, each tested twice for a same condition. Multiple curves of normalized acidification rate obtained through the multiple replications would then be averaged to represent a response from a cell capsule under a certain condition. For all microphysiometer data, each capsule was represented as the mean of all trials, however, only the number of capsules tested was used as a trial number in statistical tests. In iodide efflux studies, data was obtained from 35 mm cell

culture dishes and each dish of cells was used as a single trial number. The data were again represented as average curves of all trials for a single experimental condition.

RESULTS

1. Iodide Efflux

In testing the effect of inhibitors (glibencamide and DPC) and an activator (forskolin) of CFTR as well as an inhibitor of anion exchangers (DIDS), studies using iodide (anion) efflux were performed (Table 1). As described above, cells were grown in 35 mm cell culture dishes and left in an iodide-containing buffer to allow for equilibration of extracellular and intracellular iodide concentrations. Following the loading period, the cells were removed from the incubator and iodide efflux through CFTR was measured using the complete sample replacement technique of Venglarik et al. (1990).

1.1. CFTR Activation Increased Iodide Efflux Rates

The cAMP elevating agent, 10 μM forskolin, elicited a significant increase in iodide efflux in wild type cells (transfected with wild type CFTR), when compared to mutant (transfected with ΔF508 CFTR) and control (vector transfected) cells (Figure 5). Neither mutant or control cells exhibited any significant increase in iodide efflux (Figure 5). Forskolin activates CFTR by causing an increase in $[cAMP]_i$, thereby activating the PKA pathway and (presumably) phosphorylating the R domain of the CFTR protein (Rommens et al., 1991). Looking at iodide efflux rate (change in concentration over time) of wild type cells before stimulation (at 2.5 minutes) and at the second time point during forskolin stimulation (at 3.5 minutes), 10 μM forskolin caused about 3.5

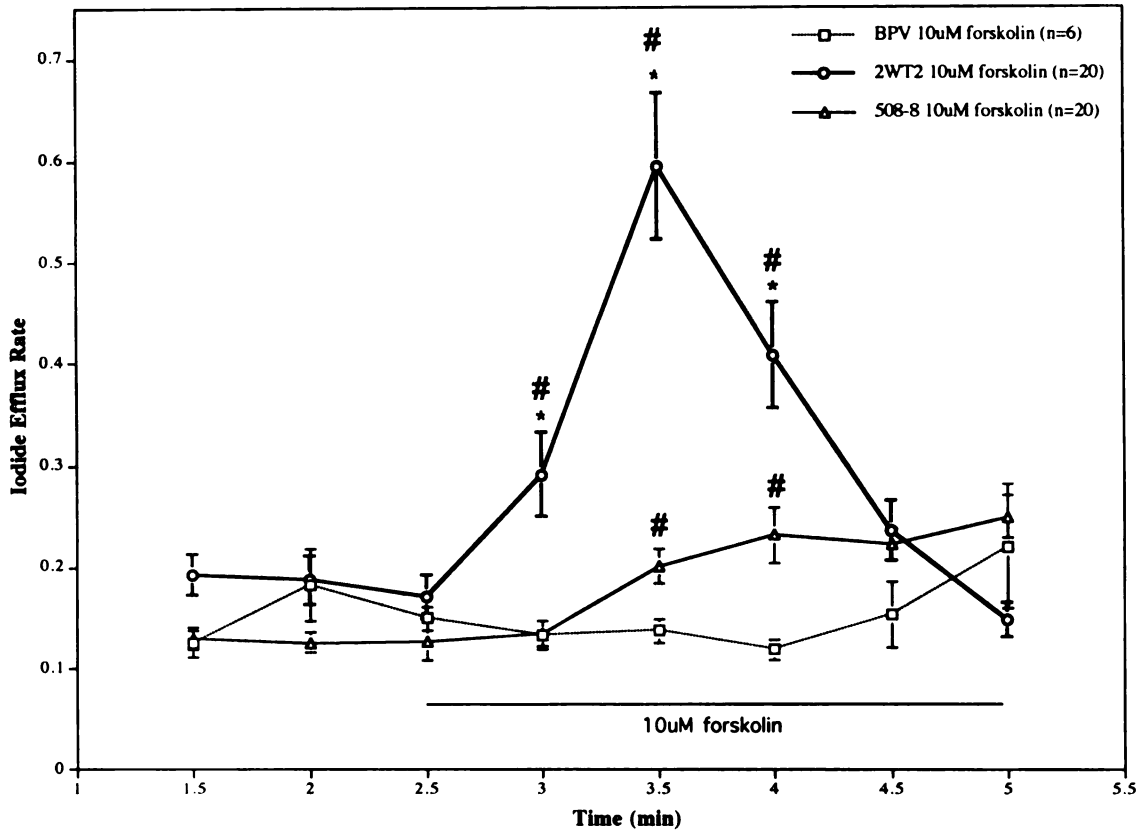

Figure 5. Effect of 10 μM forskolin treatment on iodide efflux rate of control cells transfected with vector alone (BPV), and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). When compared to mutant and control cells, 10 μM forskolin (cAMP elevating agent) exposure elicited a rapid and significant increase in iodide efflux in wild type cells. Forskolin caused a total change in rate (change in concentration over time) of 0.42 (wild type), 0.07 (mutant) and -0.01 (control) in between 2.5 minutes (before stimulation) and 3.5 minutes (second time point of forskolin treatment). Three and half fold rapid increase in rate of iodide efflux indicated an increase in CFTR activation in wild type cells, and lack thereof, in both mutant and control cells. Each point represents an average of 20 experiments for wild type and mutant cell lines and six experiments for the control cell line (* $P < 0.01$ compared to 508-8; # $P < 0.01$ compared to BPV; error bars as SEM).

fold increase in efflux rate and yielded a change in rate of 0.42 (Figure 6). Wild type cells not receiving forskolin stimulation had a slight increase between those same points, causing a non-significant change in rate of 0.08 (Figure 6). This minor drift in iodide efflux (observed in non-stimulated cells) was, however, significantly different from the much higher forskolin-elicited response. By contrast, forskolin caused only a slight increase in iodide efflux from mutant cells (equaling to a total of 0.07 change in rate between 2.5 and 3.5 minute points) and no change in control cells (Figure 7; Figure 8). The iodide efflux rate of mutant cells stimulated with 10 μM forskolin was comparable to the background noise level of non-stimulated wild type cells (Figure 6; Figure 7). Similarly, mutant and control cells that did not receive forskolin stimulation did not have any significant increase in efflux (Figure 7; Figure 8).

1.2. Anion Exchanger (AE) Inhibition Does Not Decrease Iodide Efflux Rates

Simultaneous treatment of wild type cells with 500 μM DIDS (AE inhibitor) and 10 μM forskolin resulted in a similar mean iodide efflux response to that seen with forskolin alone (Figure 9A). This time the efflux increased about 6.5 fold when compared to the pretreatment state (in between 2.5 and 3.5 minutes) and yielded a change in rate of 0.46 (Figure 9A). The mean increase in efflux rate of wild type cells treated with both DIDS and forskolin was higher (though not significantly different) than the mean increase in wild type cells treated only with forskolin (3.5 fold increase) (Figure 9A). Due to an observed unequal pretreatment iodide efflux rate, the figure was redrawn representing only those

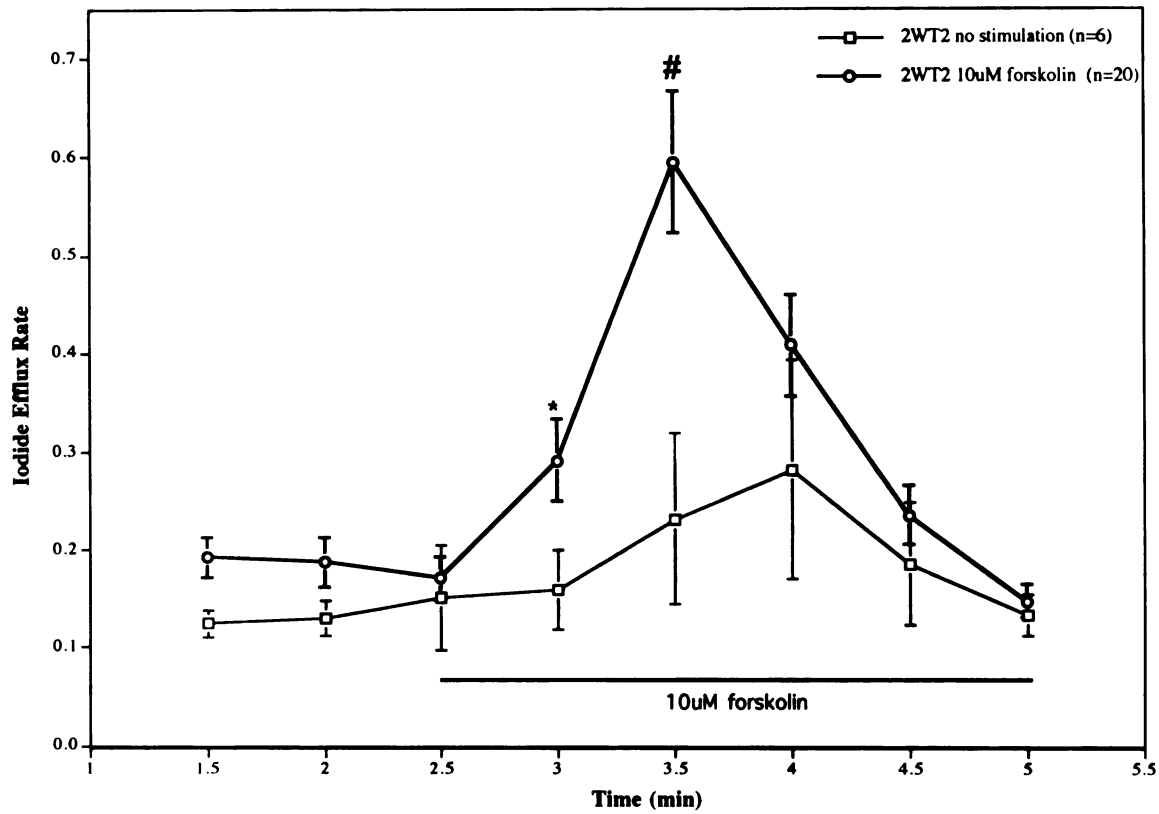

Figure 6. Effect of 10 μM forskolin treatment on iodide efflux rate of cells stably expressing wild type CFTR (2WT2). Treatment with 10μM forskolin elicited a rapid and significant increase in iodide efflux in wild type cells thus indicating functional and active CFTR. Comparing the time points (t) prior to stimulation (t=2.5) with the second time point during forskolin stimulation (t=3.5) a total change in rate of 0.42 (3.5 fold increase) was observed. Cells not receiving any drug stimulation had a slight increase between those same points, causing a total change in rate of efflux of 0.08 that was deemed insignificant and part of background level. Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for control studies with efflux buffer alone (* $P < 0.05$; # $P < 0.01$; error bars as SEM).

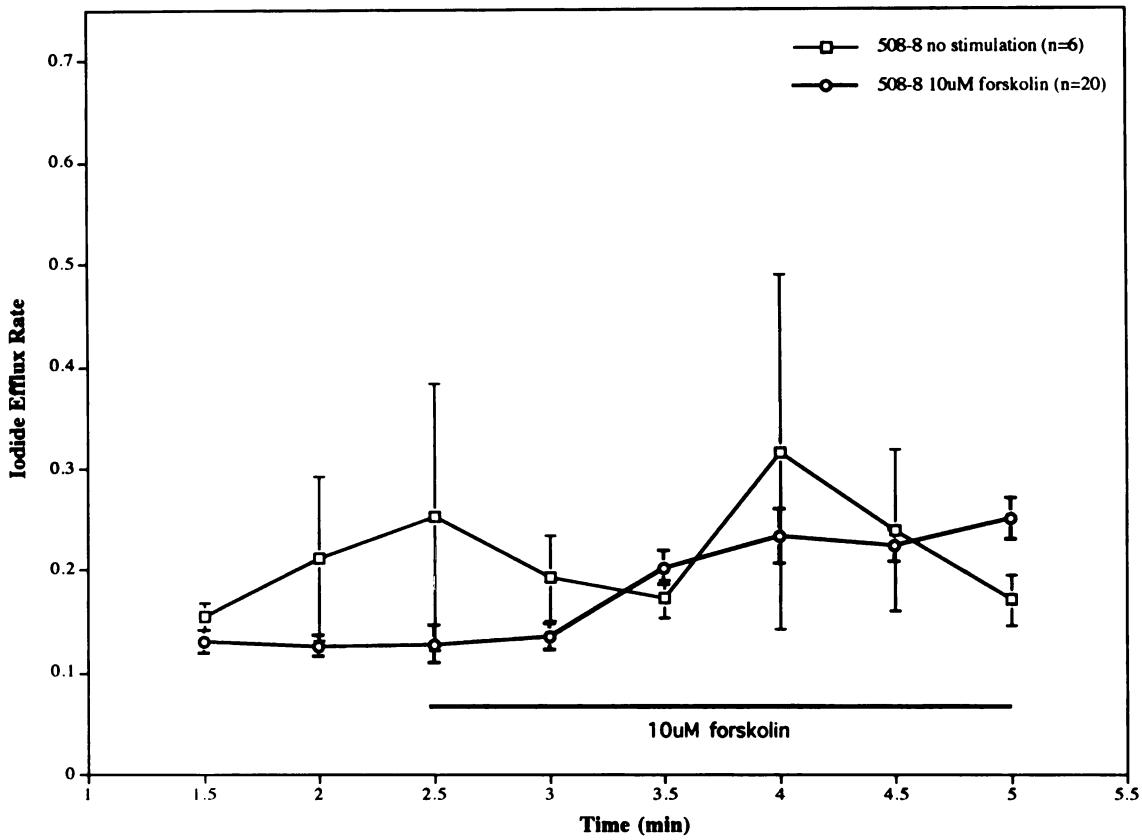

Figure 7. Effect of 10 μM forskolin treatment on iodide efflux rate of cells stably expressing ΔF508 mutant CFTR (508-8). When compared to cells not receiving treatment, application of 10 μM forskolin did not cause any significant change in rate of iodide efflux. The rate of efflux varied over time but did not indicate functional membrane expressed CFTR. Background drift was actually comparable to un-stimulated wild type cells. Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for control studies with efflux buffer alone (error bars as SEM).

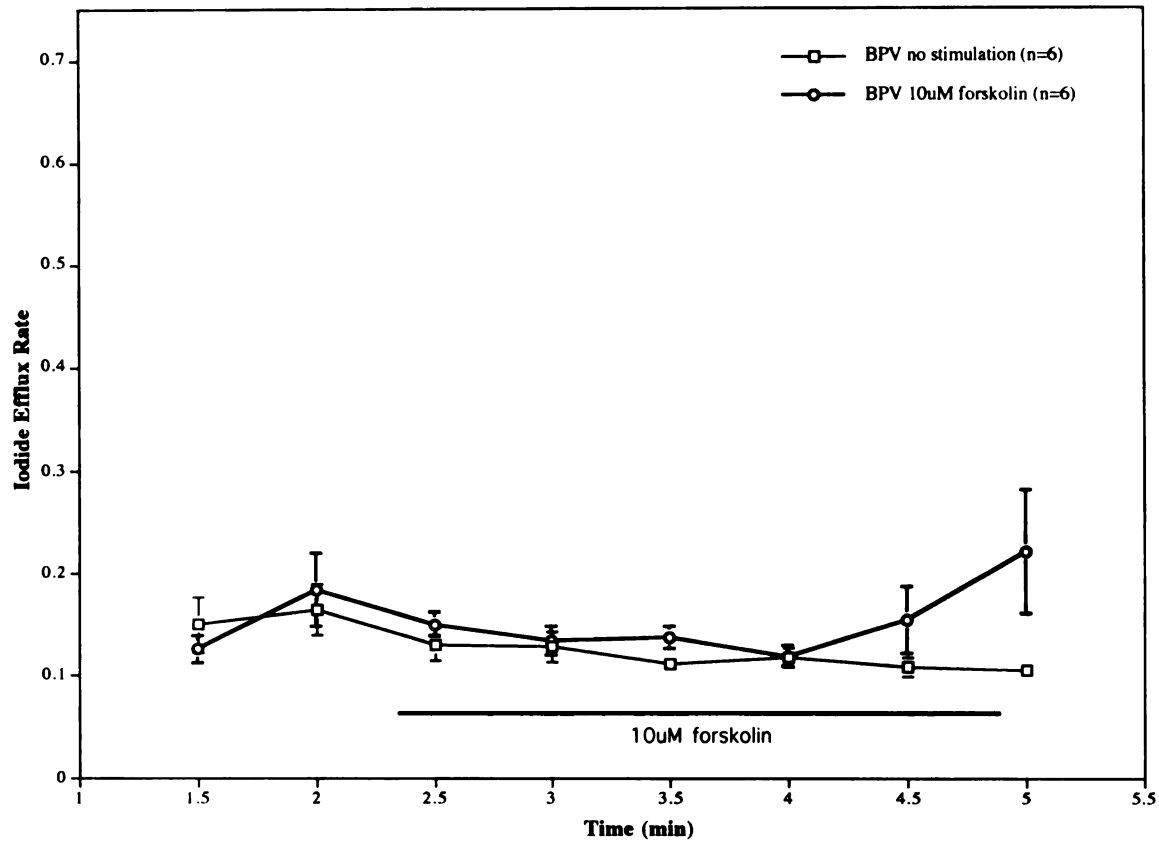

Figure 8. Effect of 10 μM forskolin treatment on iodide efflux rate of cells transfected with vector alone (BPV). Treatment of control cells with 10 μM forskolin did not cause any significant change in rate of iodide efflux. The rate of efflux remained steady for the duration of the experiment (almost identical with un-stimulated control cells) indicating no functional membrane expressed CFTR. Each point represents an average of six experiments (error bars as SEM).

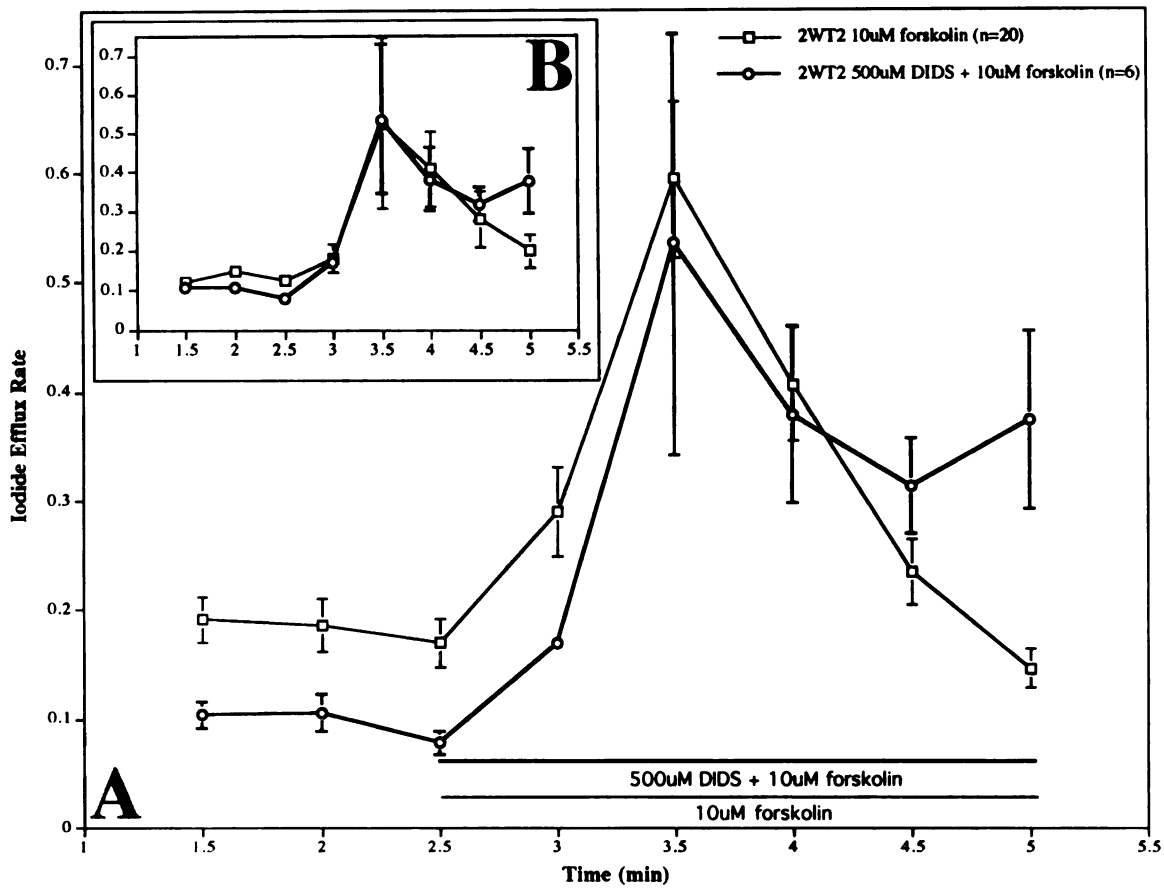

Figure 9. Effect of 500 μM DIDS and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing wild type CFTR (2WT2). (A.) 500 μM DIDS (an AE inhibitor) and 10 μM forskolin treatment of wild type cells resulted in an almost identical iodide efflux rate as that elicited by treatment with 10 μM forskolin alone. The increase in rate was about 6.5 fold as compared to the pretreatment state (in between t=2.5 and t=3.5 minutes) and yielded a change in rate of 0.46. The increase in efflux rate of wild type cells treated with both DIDS and forskolin was higher (though not statistically significantly different) than in cells treated only with forskolin (mean 3.5 fold increase). Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for studies with 500 μM DIDS and 10 μM forskolin (error bars as SEM). **(B.)** Due to differences in iodide efflux rates prior to drug treatment, a graphical comparison was created representing only those experiments performed simultaneously. The iodide efflux rates of the treatments were more similar, indicating no difference between the two treatments. The pretreatment state paralleled each other as well. Each point represents an average of six experiments (error bars as SEM).

experiments performed simultaneously (Figure 9B). The iodide efflux rates of the treatments were even more similar when presented in this fashion, indicating no difference between the two treatments. The pretreatment states paralleled each other as well. Mutant cells, on the other hand, did not show any significant change in iodide efflux as a result of treatment with 500 μM DIDS and 10 μM forskolin (Figure 10). Both sets of mutant cells (receiving DIDS and forskolin or only forskolin) did exhibit a small, steady increase in iodide efflux rate over time, which was found to be significantly different from each other (Figure 10). The efflux rate of these cells increased by approximately two fold for the duration of the entire treatment (2.5 to five minutes) (Figure 10).

1.3. CFTR Inhibition Reduced Iodide Efflux Rates

Application of 400 μM glibenclamide and 100 μM DPC (CFTR inhibitors) along with 10 μM forskolin, decreased iodide efflux in wild type cells indicating, at least partial CFTR inhibition (Figure 11). Glibenclamide and DPC reduced the increases in iodide efflux elicited by forskolin to only about 1.5 fold (50%) in wild type cells, causing a total change in rate of 0.12 (Figure 11). Due to data variations, however, when compared to a 3.5 fold (350%) increase in rate of efflux caused by forskolin treatment alone, reduction of increase in efflux rate of cells treated with CFTR inhibitors was found to be insignificant ($p=0.06$). The same treatment in mutant cells did not cause any significant change or difference in the overall rate of efflux in either cells treated with forskolin alone, or treated with both forskolin and glibenclamide and DPC (Figure 12). Both treatments did,

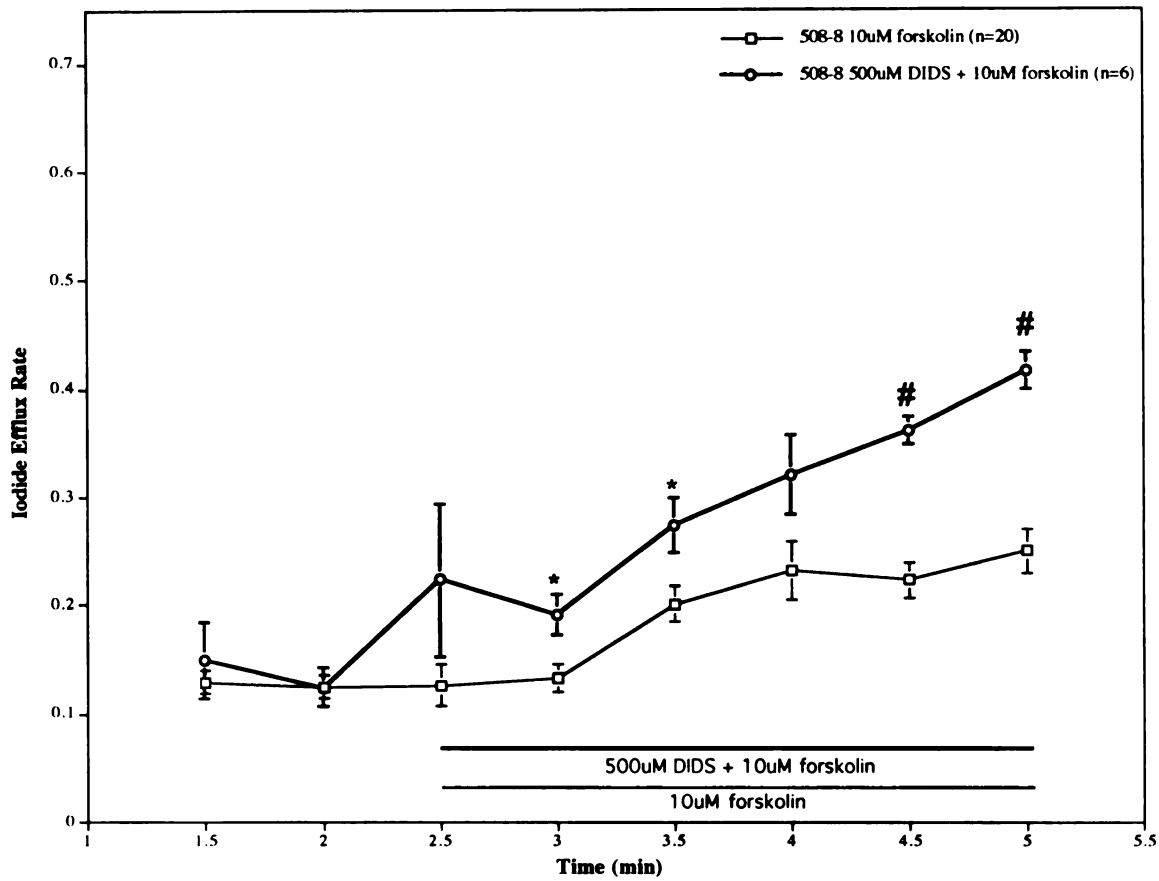

Figure 10. Effect of 500 μM DIDS and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing ΔF508 mutant CFTR (508-8). Mutant cells did not show a sudden increase in iodide efflux as a result of treatment with 500 μM DIDS and 10 μM forskolin as did wild type cells (Figure 9). Both sets of mutant cells (receiving DIDS and forskolin or only forskolin) did exhibit a parallel, steady increase. Even though the differences in mean efflux rates were statistically significant, the efflux rates increased equally, by only two fold for the duration of the entire experiment (2.5 to five minutes) in both drug treatments. Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for studies with 500 μM DIDS and 10 μM forskolin (* $P < 0.05$; # $P < 0.01$; error bars as SEM).

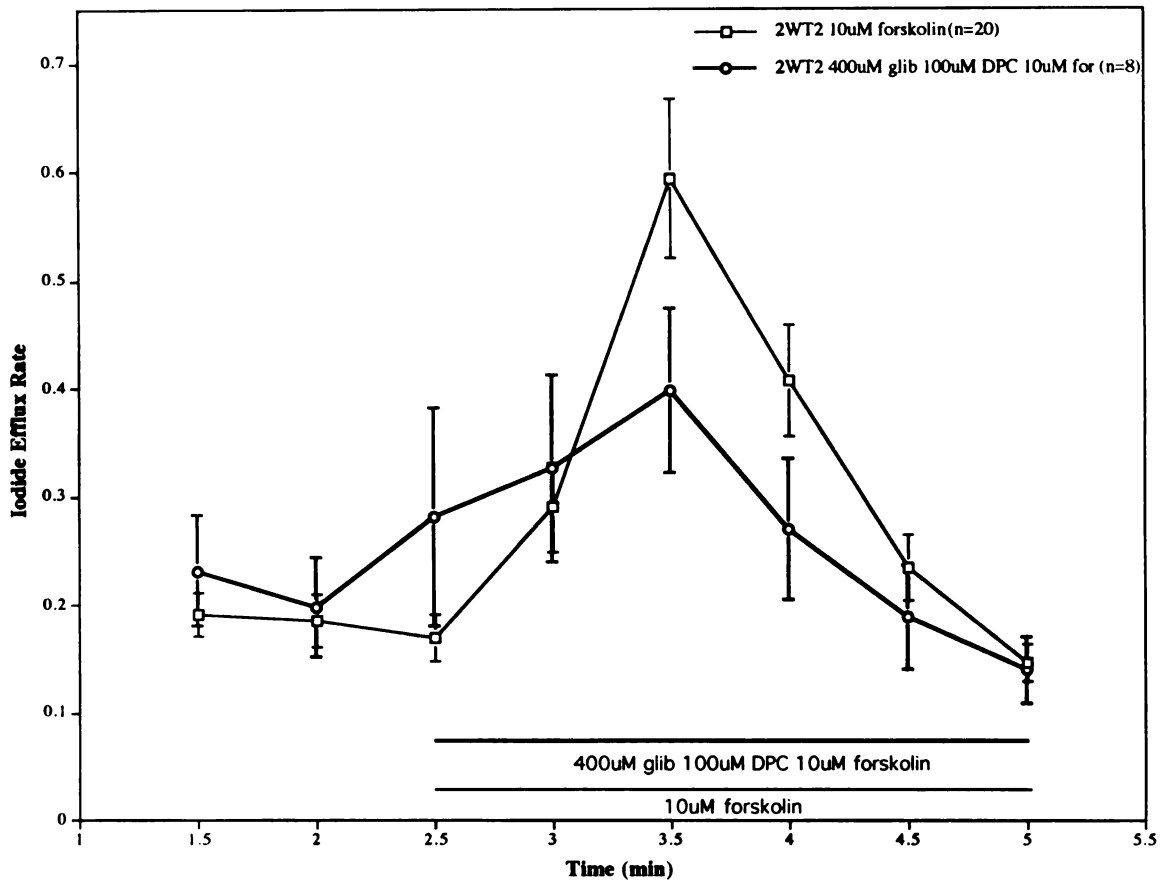

Figure 11. Effect of 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing wild type CFTR (2WT2). Application of 400 μM glibenclamide, 100 μM DPC (CFTR inhibitors), and 10 μM forskolin decreased iodide efflux in wild type cells, indicating partial CFTR inhibition. Treatment with CFTR inhibitors and forskolin caused a maximum 1.5 fold (50%) increase in efflux rate of wild type cells causing a total change in rate of efflux of 0.12 (between t=2.5 and t=3.5). Due to large data variations, when compared to a 3.5 fold (350%) increase in rate of efflux caused by forskolin treatment alone, the mean increase in efflux rate was not found to be significant (p=0.06). Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for studies with 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin (error bars as SEM).

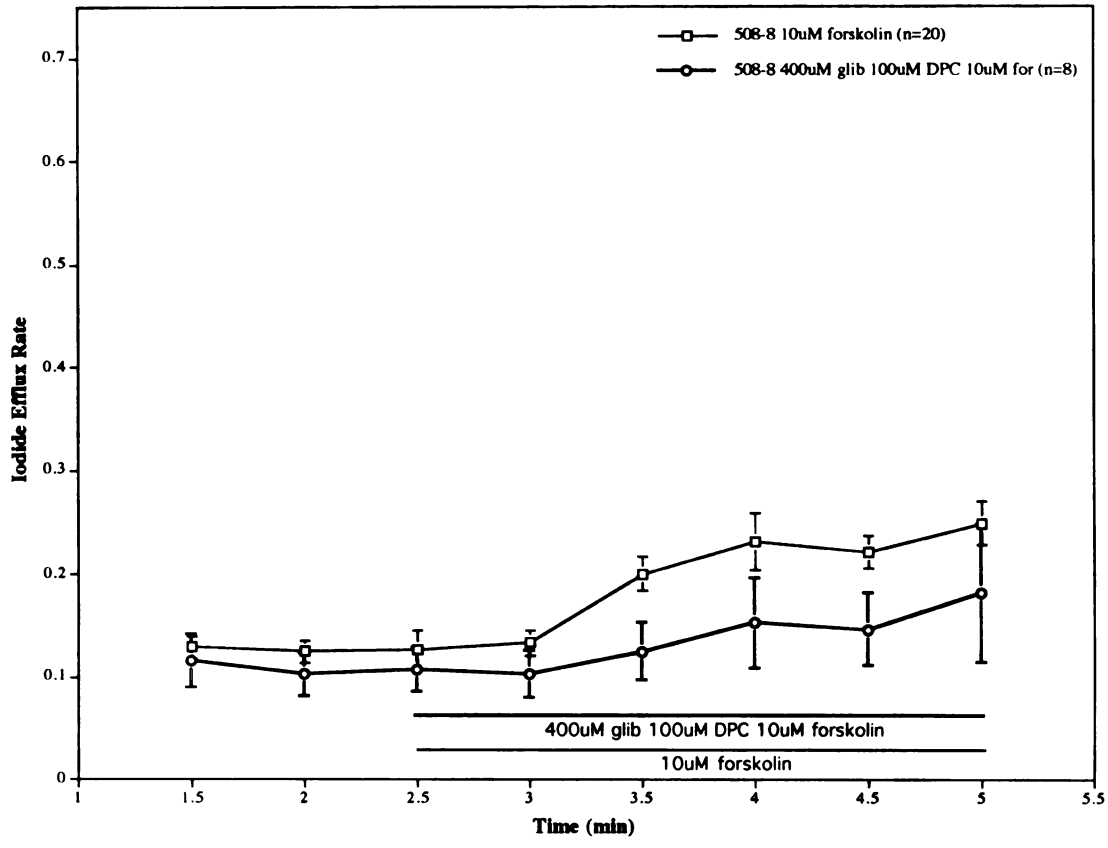

Figure 12. Effect of 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing ΔF508 mutant CFTR (508-8). Treatment of mutant cells with 400 μM glibenclamide, 100 μM DPC (CFTR inhibitor cocktail) and 10 μM forskolin did not cause any significant change in the overall rate of efflux in either cells treated with forskolin alone, or treated with inhibitor cocktail and forskolin. Both drug treatments did exhibit a parallel, steady increase for the duration of the experiment (in between t=2.5 and t=5). Glibenclamide, DPC and forskolin treatment caused only about a 1.7 fold (70%) increase in the rate of efflux, while forskolin treatment alone caused about a two fold increase (100%). Their differences in rates were not overall statistically significant (p=0.24 at t=3; p=0.02 at t=3.5; p=0.13 at t=4; p=0.06 for at t=4.5). Each point represents an average of 20 experiments for 10 μM forskolin treatment and six experiments for studies with 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin (error bars as SEM).

however, cause a steady increase in efflux rate for the duration of the experiment (1.7 fold increase with forskolin alone and two fold increase with inhibitors and forskolin) (Figure 12). No significant difference between the two treatments was observed.

If CFTR inhibition occurred, wild type cells treated with CFTR inhibitors (400 μM glibenclamide and 100 μM DPC) and the stimulator (10 μM forskolin) were expected to behave similarly to mutant cells treated with a stimulator. A significant difference was, however, observed between the two treatments. Wild type cells exhibited a rate of efflux increased by about 50%, causing a change in rate of efflux by 0.12, while mutant cells showed a change in rate of only 0.07, thus again indicating only partial inhibition of CFTR (Figure 13).

1.3.1 Vehicle Controls Studies for CFTR Inhibition

A last set of iodide efflux studies were carried out as a vehicle control for 0.4% DMSO. Studies were performed where cells were treated with 0.4% DMSO and forskolin and compared to DMSO treatment alone. In wild type cells, 0.4% DMSO and 10 μM forskolin treatment caused a significant increase in iodide efflux rate, increasing 5.5 fold (causing a 0.87 increase in change of rate) (Figure 14). The same DMSO and forskolin treatment in mutant cells did not cause any significant change and remained rather steady for the duration of the experiment (Figure 15). When compared to the wild type treatment it was significantly lower (Figure 16). The 0.4% DMSO treatment alone did not cause any significant change in iodide efflux rate in either wild type or mutant cell lines (Figure 14;

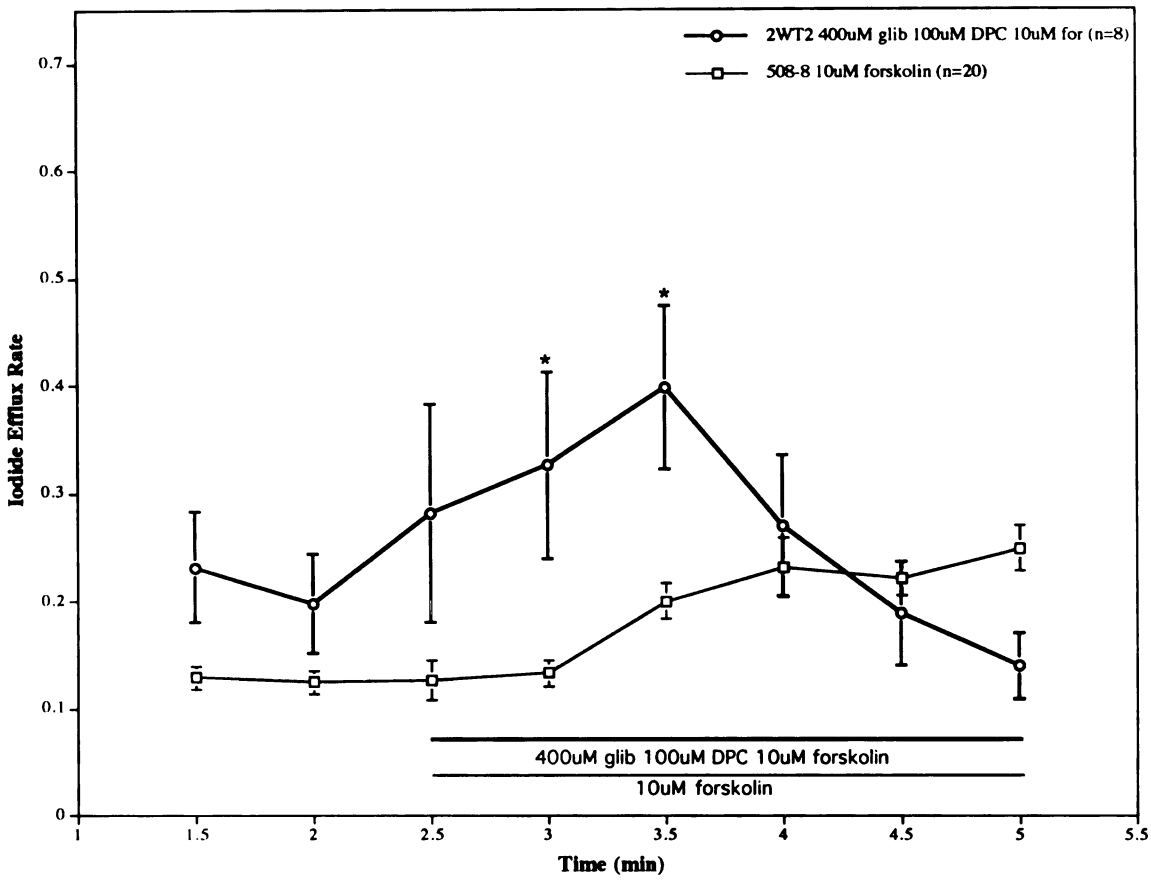

Figure 13. Comparison of CFTR inhibition in cells stably expressing wild type CFTR (2WT2) with CFTR activation in cells stably expressing ΔF508 mutant CFTR (508-8). Wild type cells treated with CFTR inhibitors (400 μM glibenclamide and 100 μM DPC) and stimulator (10 μM forskolin) exhibited an increase when compared to mutant cells treated with forskolin. Wild type cells exhibited a rate of efflux increased by about 50% and causing a change in rate of efflux by 0.12, while mutant cells showed a change in rate of only 0.07 (between t=2.5 and t=3.5). If complete CFTR inhibition had occurred, it would be expected that these two treatments would elicit the same results. Each point represents an average of 20 experiments for mutant cells and eight experiments for wild type cells (* $P < 0.05$; error bars as SEM).

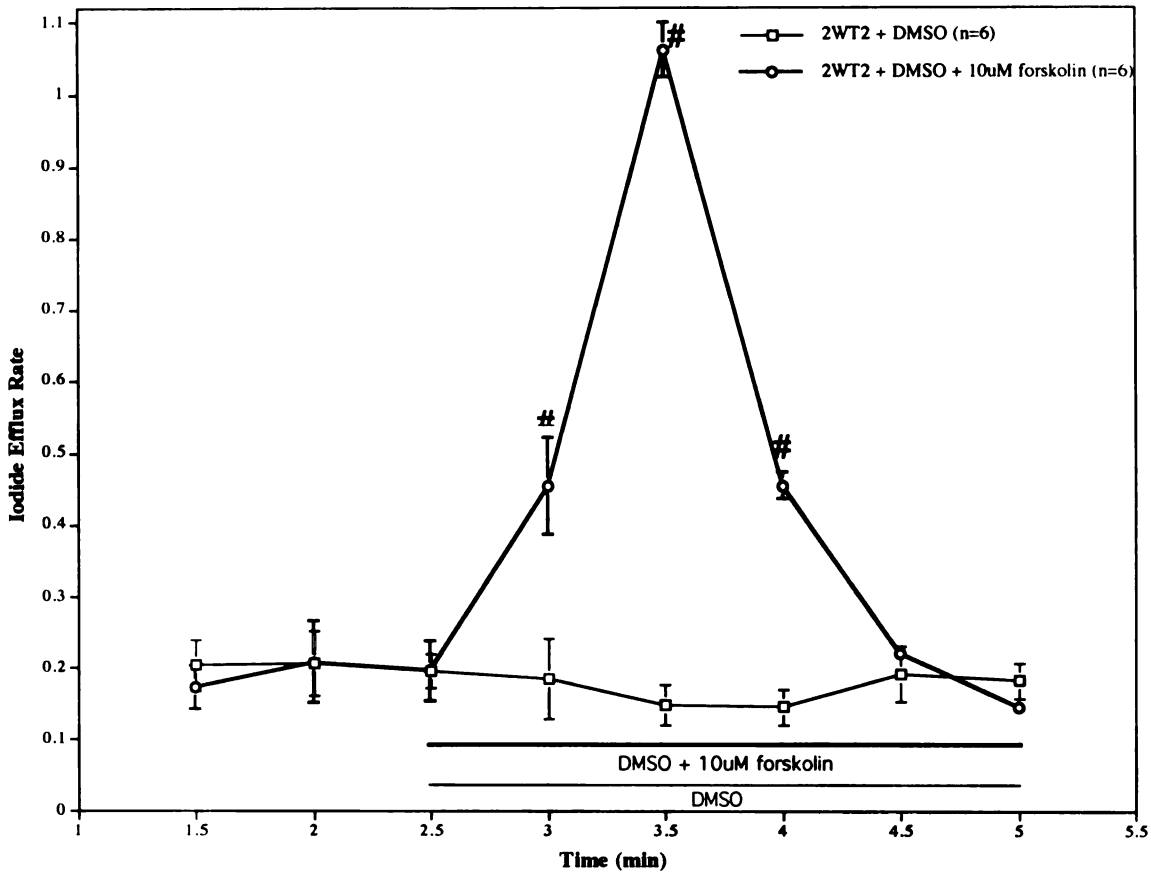

Figure 14. Effect of 0.4% DMSO and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing wild type CFTR (2WT2). DMSO studies were performed as a vehicle control for CFTR inhibitor experiments. Wild type cell treatment with 0.4% DMSO and 10 μM forskolin caused a significant increase in iodide efflux rate of 5.5 fold (0.87 increase in rate of efflux) in between t=2.5 and t=3.5. This forskolin-elicited response was higher than the 3.5 fold increase observed in wild type cells that did not receive DMSO (Figure 6.) The 0.4% DMSO treatment alone did not cause any significant change in iodide efflux rate. Each point represents an average of six experiments. (# $P < 0.01$; error bars as SEM)

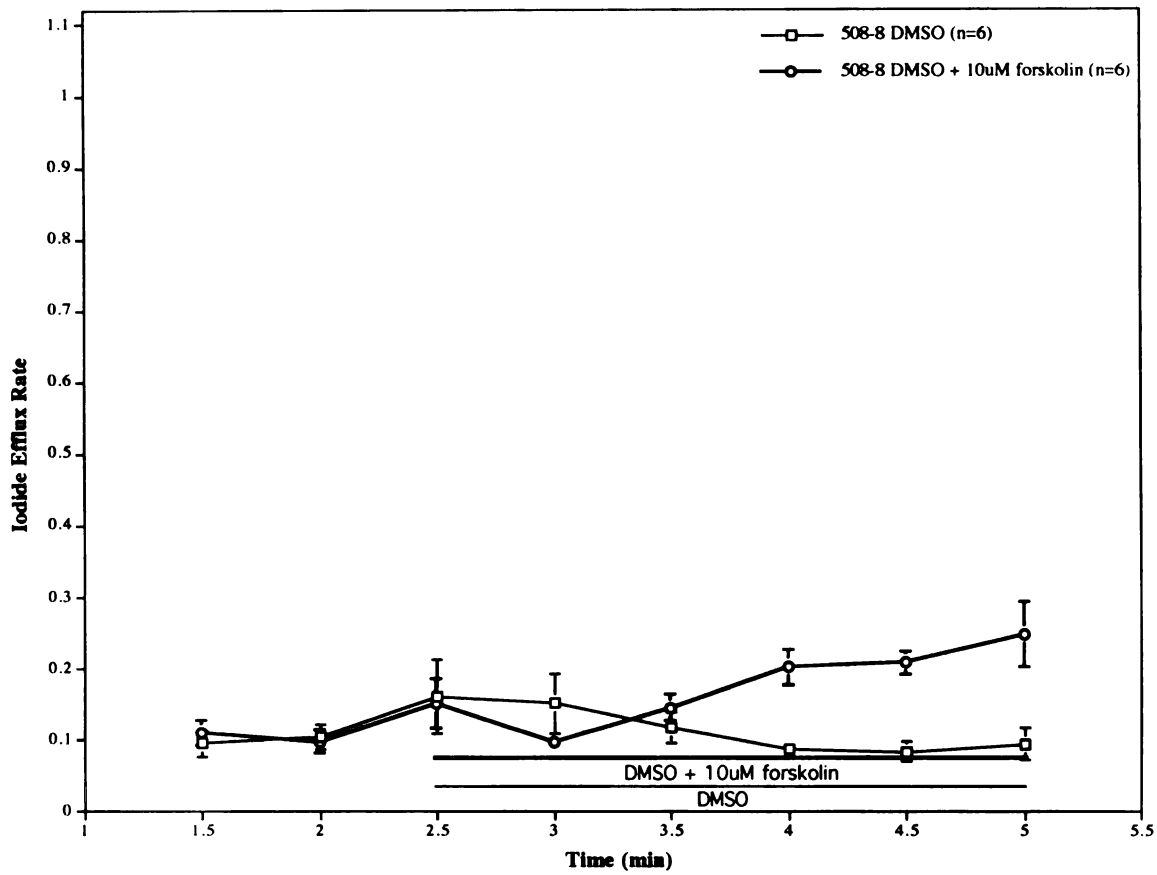

Figure 15. Effect of 0.4% DMSO and 10 μM forskolin treatment on iodide efflux rate of cells stably expressing ΔF508 mutant CFTR (508-8). Mutated cells treated with 0.4% DMSO and 10 μM forskolin did not exhibit any significant change in rate of iodide efflux for the duration of the trial. No significant change in efflux was noted with mutant cells treated with 0.4% DMSO alone. Each point represents an average of six experiments (error bars as SEM).

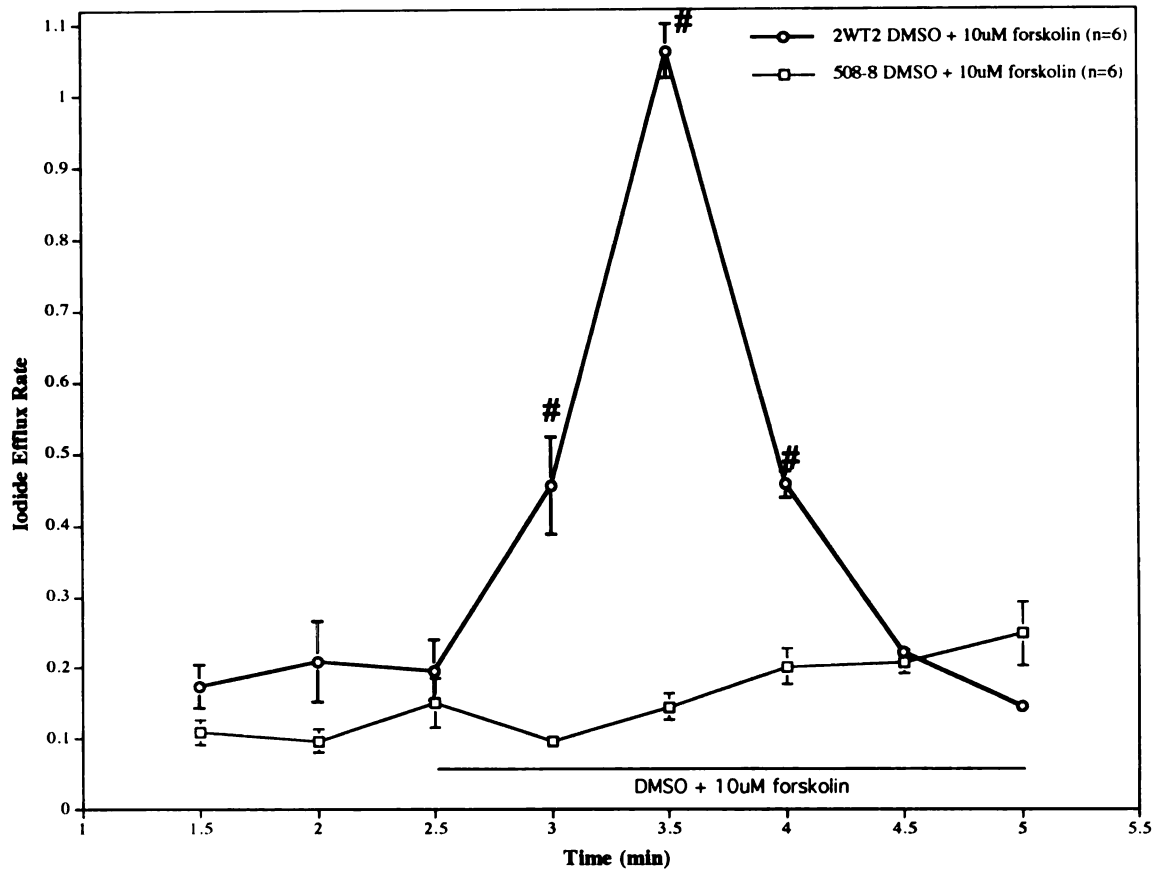

Figure 16. Comparison of CFTR stimulation with 0.4% DMSO and 10 μM forskolin treatment in cells stably expressing wild type CFTR (2WT2) and ΔF508 mutant CFTR (508-8). Wild type cells treated with 0.4% DMSO and 10 μM forskolin exhibited a significant increase in iodide efflux rate of 5.5 fold. The same treatment in mutant cells did not cause any significant change in efflux rate. Each point represents an average of six experiments (# $P < 0.01$; error bars as SEM).

Figure 15).

2. Extracellular Acidification Rates

The microphysiometer was used to detect changes in extracellular pH (pH_o) as a result of alterations in metabolic rate, changes in intracellular pH and transient acid/base fluxes such as those caused by short-lived transporter activity. In our studies, cells were perfused with physiological buffers containing various stimulators and inhibitors of CFTR and anion exchangers (glibenclamide, forskolin, Cl^- free medium and DIDS). The resulting pH_o was monitored via microphysiometer technology to characterize bicarbonate conductance through CFTR and to examine the possibility of CFTR serving as a Cl^-/HCO_3^- exchanger (Table 1).

2.1. CFTR Activation Decreases Extracellular Acidification Rates

It has been shown that forskolin causes increased extracellular alkalinization of CFTR-expressing cells and increased extracellular acidification of CFTR-deficient control cells (Luckie et al., 2001). In the current studies, we compared acidification rates of cells transfected with vector alone (control), and of cells stably expressing wild type CFTR or mutated CFTR (wild type and mutant, respectively). A ten-minute exposure to 10 μM forskolin caused decreased acidification rate in wild type cells when compared to mutant and control cells (Figure 17). An initial increase in acidification rate for mutant (90%), wild type (35%) and control cells (35%) was observed. Four minutes following

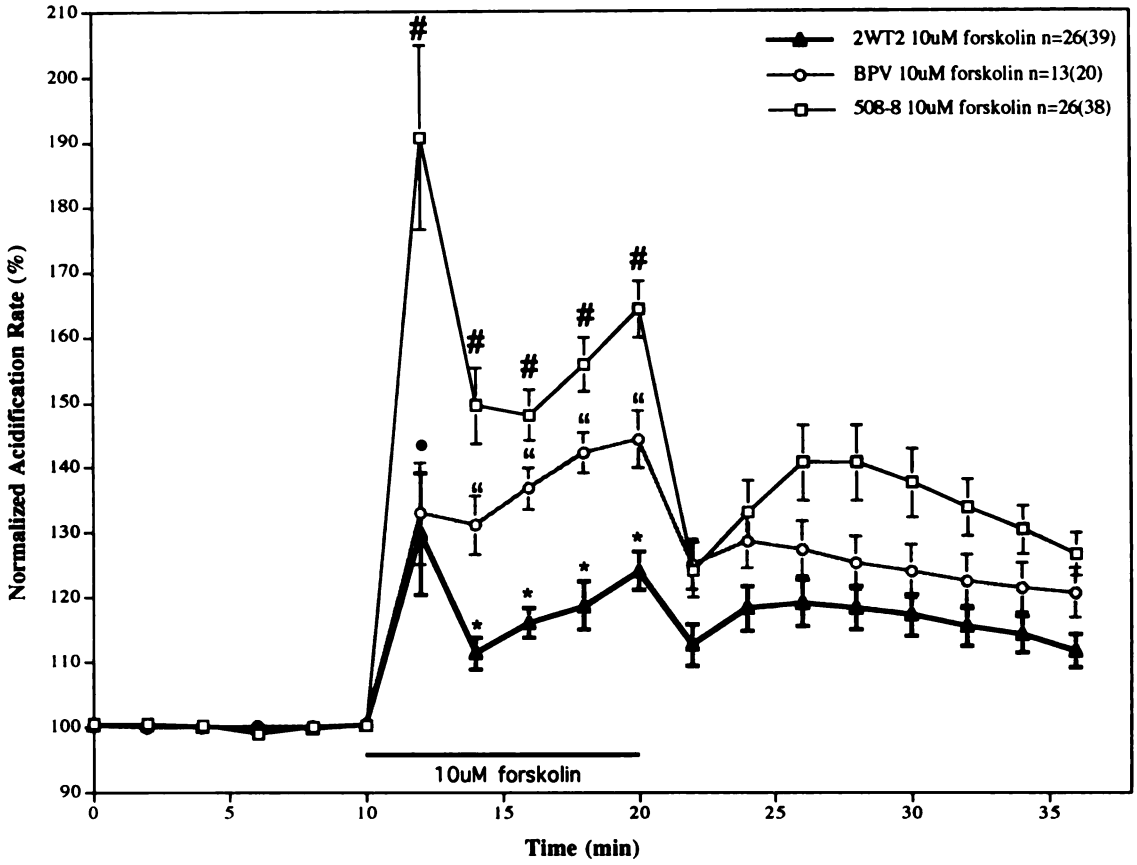

Figure 17. Effect of 10 μM forskolin treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). A ten-minute exposure to 10 μM forskolin caused decreased acidification rate in wild type cells when compared to mutant and control cells. An initial increase in acidification rate for mutant (90%), wild type (35%) and control cells (35%) was observed. Four minutes following the exposure to forskolin, acidification rate for mutant cells was reduced to 50% compared to the pretreatment state. Acidification rate remained high for the duration of the treatment and further increased to about 65%. Acidification rate of control cells continued to rise to final level of 45%. Acidification rate of wild type cells was reduced to approximately 25% and was significantly lower than the other two cell lines for the duration of the forskolin exposure. Even after the forskolin washout, acidification rate of all three cell lines remained separated from each other. Each point represents an average of n=26(39) for wild type cells, n=26(38) for mutant cells, and n=13(20) for control cells (* $P < 0.01$ compared to 508-8 and BPV; # $P < 0.01$ compared to 2WT2 and BPV; " $P < 0.01$ compared to 508-8 and 2WT2; • $P < 0.01$ compared to 508-8; error bars as SEM).

the exposure to forskolin, acidification rate for mutant cells was reduced and was now increased by only 50% compared to the pretreatment state. Acidification rate in these cells remained higher for the duration of the treatment and was even further increased to a final value of about 65%. Acidification rate of control cells increased and continued to rise to a final 45% increase, while the rate in wild type cells decreased and was significantly lower than the other two cell lines for the duration of forskolin exposure. Wild type cells' final acidification rate increase, as compared to the pretreatment state, was only 25%. They exhibited a distinctly different acidification rate from both control and mutant cell lines. Even after the forskolin washout, acidification rate of all three cell lines remained different from each other.

2.2. AE Inhibition Decreases Extracellular Acidification Rates

A general anion exchange (AE) inhibitor DIDS was tested for its ability to inhibit CFTR. Treatment with 500 μM DIDS alone in wild type cells resulted in decreased acidification rate by about 25% (Figure 18). When this treatment was combined with 10 μM forskolin, the effect was amplified yielding a 55% decrease in acidification rate, suggesting a still functional HCO_3^- efflux (Figure 19). DIDS treatment alone in control cell lines also elicited a decrease in acidification rates, by as much as 150% (Figure 18). The same treatment in mutant cell lines caused a less consistent reponse, however, it did exhibit an overall pattern of decreased acidification rate (Figure 18). When DIDS was combined with 10 μM forskolin treatment, mutant and control cell lines showed decreased acidification rates in

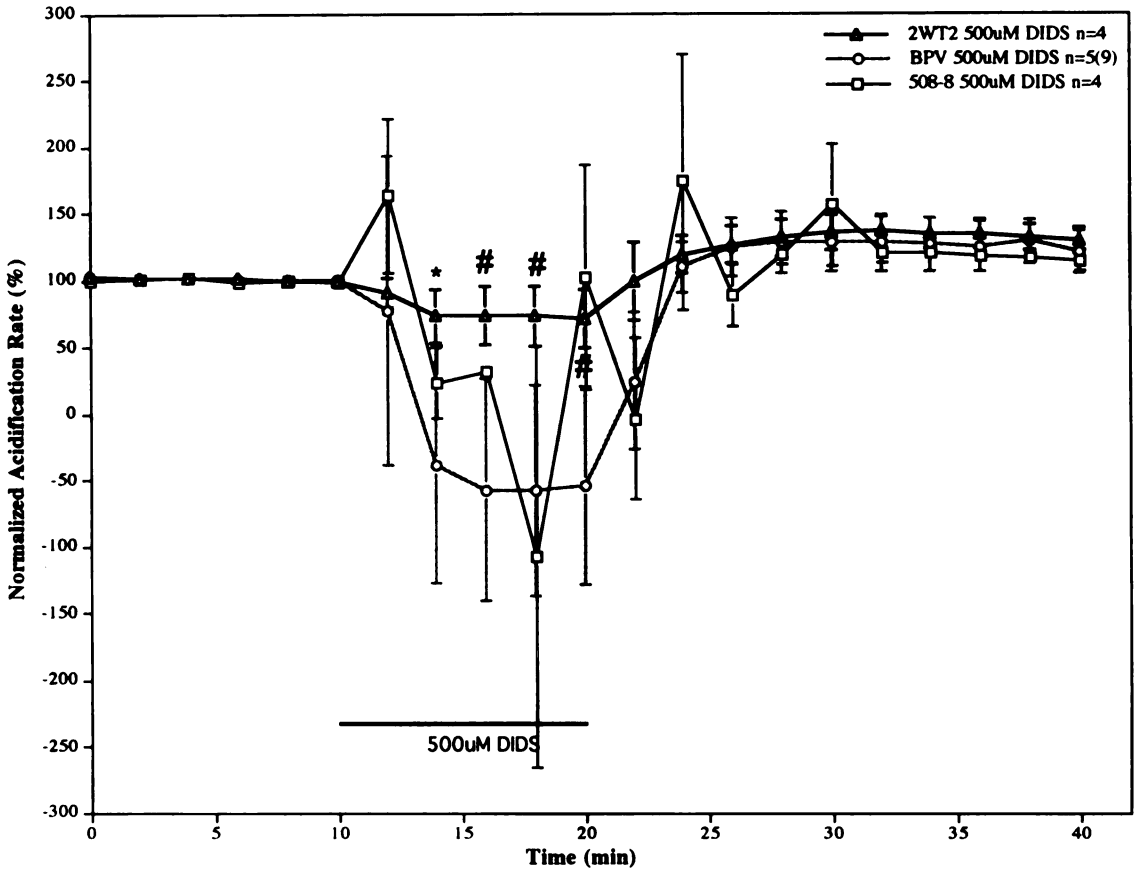

Figure 18. Effect of 500 μM DIDS treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). Treatment with the general AE inhibitor (500 μM DIDS) in wild type cells resulted in decreased acidification rate by about 25%. In control cells, the same treatment yielded a decrease in acidification rate of 150%. The same treatment in mutant cell lines caused a less consistent reponse, however, it did exhibit an overall pattern of decreased acidification rate. Each point represents an average of n=4 for wild type and mutant cells, and n=5(9) for control cells (# $P < 0.05$ compared to BPV; * $P < 0.05$ compared to 508-8; error bars as SEM).

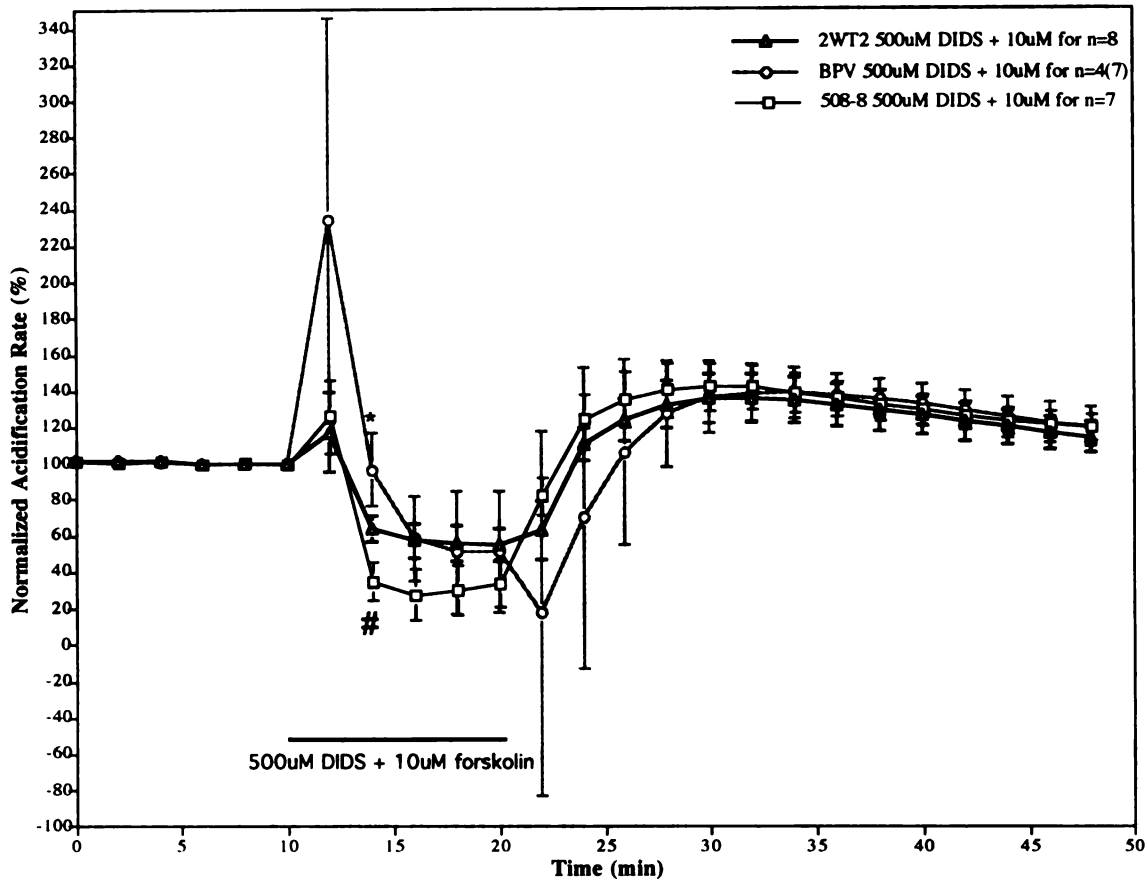

Figure 19. Effect of 500 μM DIDS and 10 μM forskolin treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). Treatment with the AE inhibitor, 500 μM DIDS, in wild type cells resulted in a decreased acidification rate of about 25% (Figure 18). However, when this treatment was combined with 10 μM forskolin the effect was amplified, yielding about a 50% decrease in acidification rate, thus suggesting still functional forskolin stimulated HCO_3^- efflux. Simultaneous DIDS and forskolin treatment in mutant and control cell lines also resulted in decreased acidification rate in the range of about 50-70%. Each point represents an average of n=8 for wild type cells, n=7 for mutant cells, and n=4(7) for control cells (# $P < 0.05$ compared to 2WT2; * $P < 0.01$ compared to 508-8; error bars as SEM).

the range of about 50-70% (Figure 19). Overall, there was not much significant difference observed between acidification rates in different cell lines (wild type, mutant and control) in any of the DIDS studies (Figure 18; Figure 19).

2.3. CFTR Inhibition Increases Extracellular Acidification Rates

We used 400 μM glibenclamide and 100 μM DPC (commonly used CFTR inhibitors) along with 10 μM forskolin to test their ability to inhibit CFTR and therefore bicarbonate efflux, thus affecting the pH_o. If CFTR is inhibited, we would expect to see an increase in acidification rates. The simultaneous inhibitor and stimulator treatment yielded about a 60-120% increase in acidification rate in all three cell lines (60% for wild type, about 90% for control and 120% for mutant cell lines) (Figure 20). When compared to only 20-25% increase in acidification rates of wild type cells with only forskolin treatment, these results (60% increase) indicate inhibition of CFTR in wild type cells. Treatment with 400 μM glibenclamide and 100 μM DPC alone in all three cell lines elicited similar results as a combined treatment with forskolin (45-100% increase in acidification rates), supporting previous indications of CFTR inhibition (Figure 21). There was no significant difference in any of the three cell lines in both studies involving 400 μM glibenclamide and 100 μM DPC (Figure 20; Figure 21).

2.4. Cl^- Free Buffer and CFTR Activation Significantly Reduce Extracellular Acidification Rates

Our final studies monitored acidification rates of cells in Cl^- free media with and

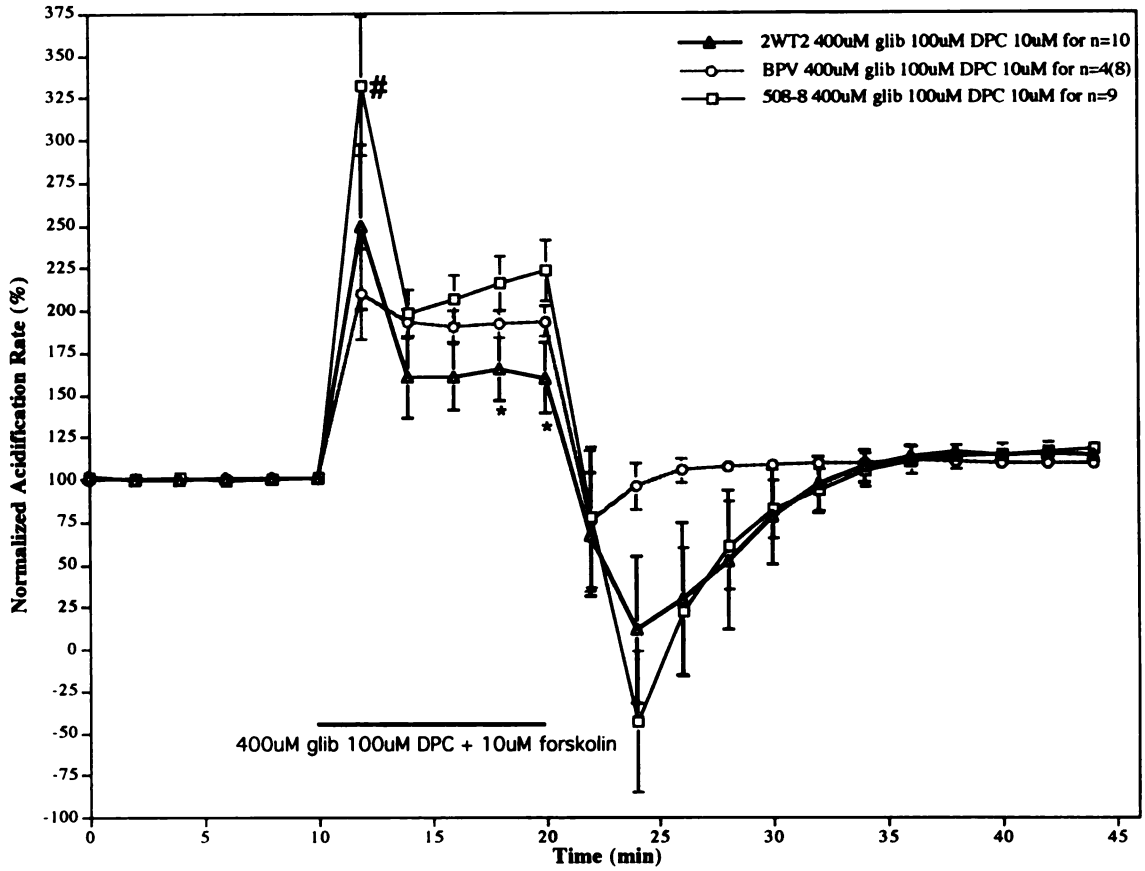

Figure 20. Effect of 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). Treatment with 400 μM glibenclamide and 100 μM DPC (CFTR inhibitors) along with 10 μM forskolin was found to cause about a 60-120% increase in acidification rate in all three cell lines (60% for wild type, and about a 90-120% for control and mutant cell lines respectively). When compared to only a 25-35% increase in acidification rates with just forskolin treatment, this increased response indicates inhibition of CFTR in wild type cells. There were no sustained differences among any of the three cell lines. Each point represents an average of n=10 for wild type cells, n=9 for mutant cells, and n=4(8) for control cells (# $P < 0.01$ compared to BPV; * $P < 0.05$ compared to 508-8; error bars as SEM).

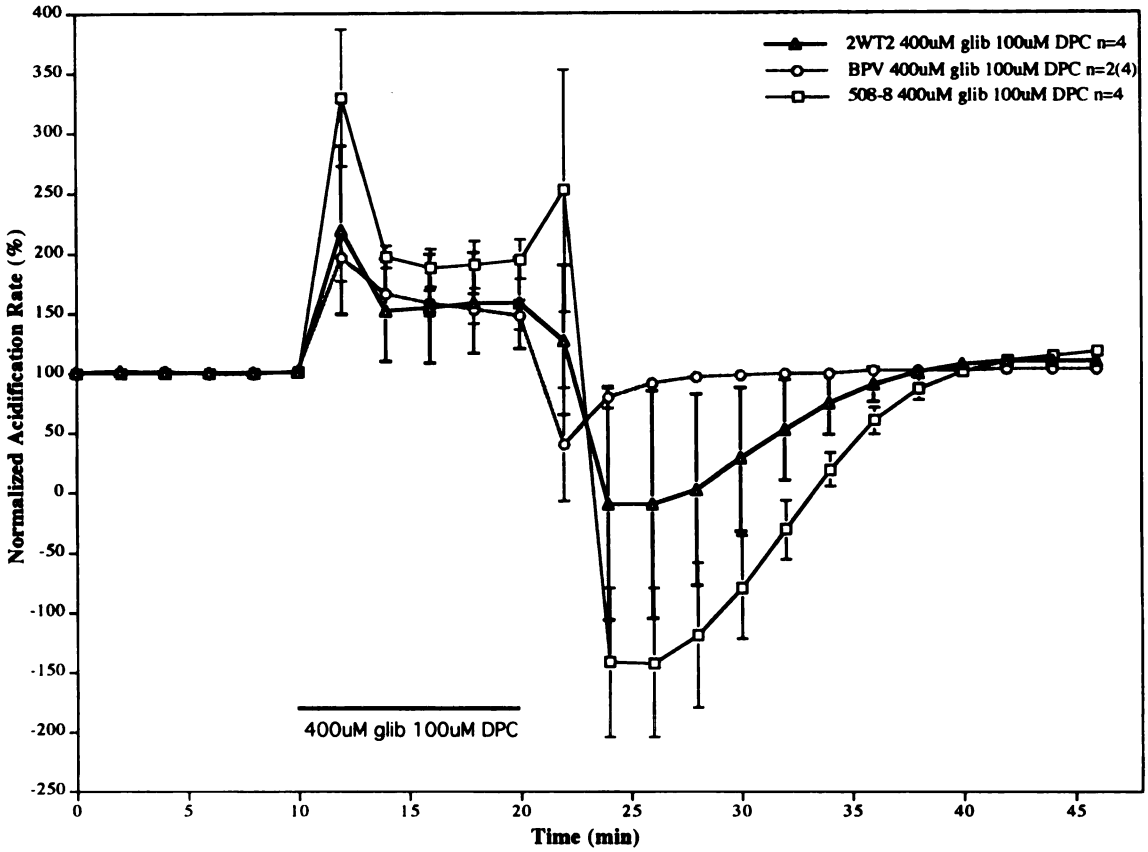

Figure 21. Effect of 400 μM glibenclamide and 100 μM DPC treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). Treatment with 400 μM glibenclamide and 100 μM DPC (CFTR inhibitors) elicited similar results to a combined treatment with forskolin in all three cell lines (45-100% increase in acidification rates) thus supporting the previous indication of CFTR inhibition. There were no statistically significant differences among any of the three cell lines. Each point represents an average of n=4 for wild type and mutant cells, and n=2(4) for control cells (error bars as SEM).

without 10 μM forskolin. By introducing a Cl^- free environment on the extracellular side, thus causing a concentration gradient for Cl^- to exit the cells and HCO_3^- to enter, we can examine the possibility that CFTR is a Cl^-/HCO_3^- exchanger. If CFTR has anion exchanger qualities, when comparing wild type cells to mutant and control cells, we would not expect to see decrease in acidification rate. HCO_3^- would only be able to enter the cell while Cl^- would be exiting the cell, thus maybe even causing a higher increase in acidification rate as compared to mutant and control cells. Following the switch to Cl^- free medium containing 10 μM forskolin, the acidification rate of wild type cells decreased by about 140% while there was no change in either mutant or control cells (Figure 22). This decrease in acidification rates of wild type cells was short lived (Figure 22). When compared to 10 μM forskolin treatment in running medium, the 10 μM forskolin in Cl^- free medium was shown to have a much higher impact on acidification rate (Figure 23). An exposure to 10 μM forskolin in running media elicited an increase in acidification rate in wild type cells of about 35% (Figure 23). Four minutes following the drug exposure acidification rate in wild type cells was reduced to a final increase, as compared to pretreatment state, of approximately 25% (Figure 23). This difference in acidification response in these two media was found to be significantly different. The data suggests continued, and even increased, alkalinization of extracellular environment in wild type cells despite the Cl^- free media.

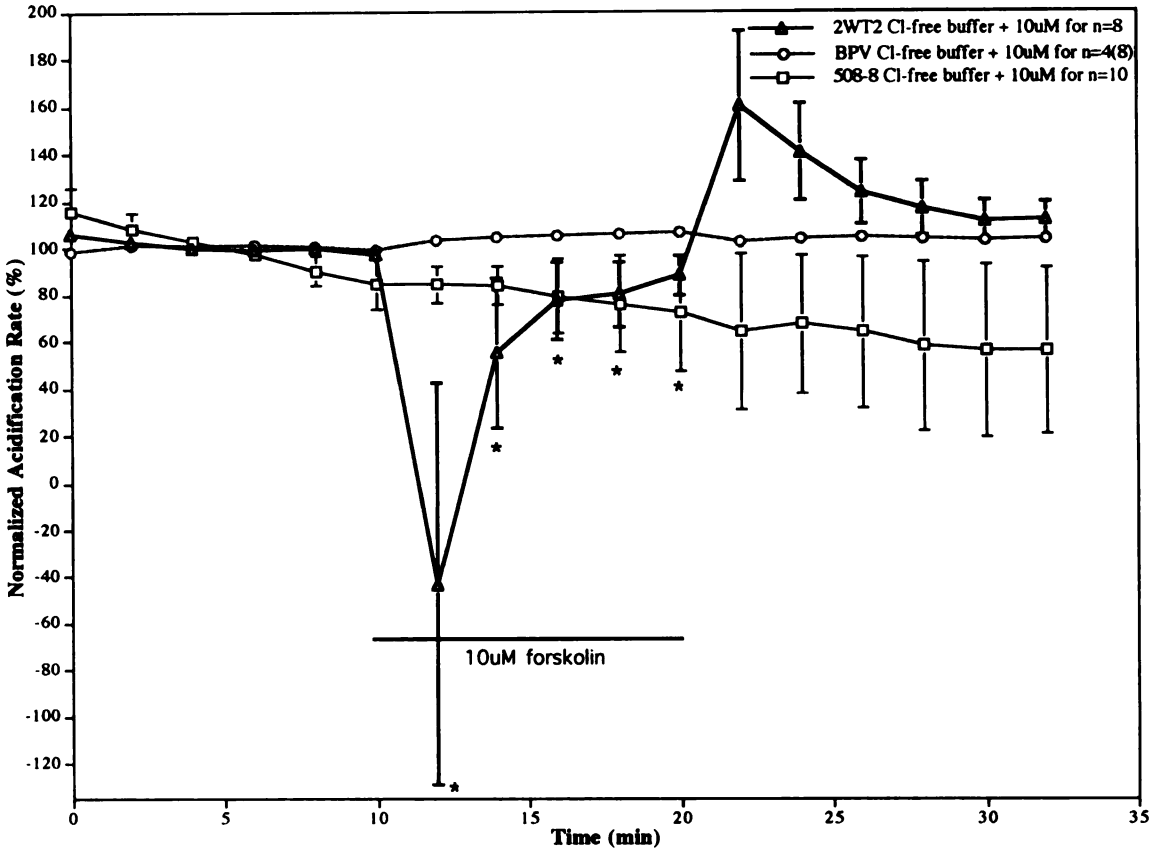

Figure 22. Effect of Cl^- free media and 10 μM forskolin treatment on normalized acidification rates of control cells (BPV) and cells stably expressing wild type CFTR (2WT2) or ΔF508 mutant CFTR (508-8). By introducing a Cl^- free extracellular environment, we examined CFTR as a possible Cl^-/HCO_3^- exchanger. During Cl^- free conditions throughout the application of 10 μM forskolin, the acidification rate of wild type cells rapidly decreased by about 140%. No such decrease was observed in either mutant or control cells. Evidence points to a continued alkalinization of the extracellular environment despite the Cl^- free media. Despite the observed differences between wild type and mutant cells, however, they were not found to be statistically significant. Each point represents an average of n=8 for wild type cells, n=10 for mutant cells, and n=4(8) for control cells (* $P < 0.01$ compared to BPV cells by both 2WT2 and 508-8; error bars as SEM).

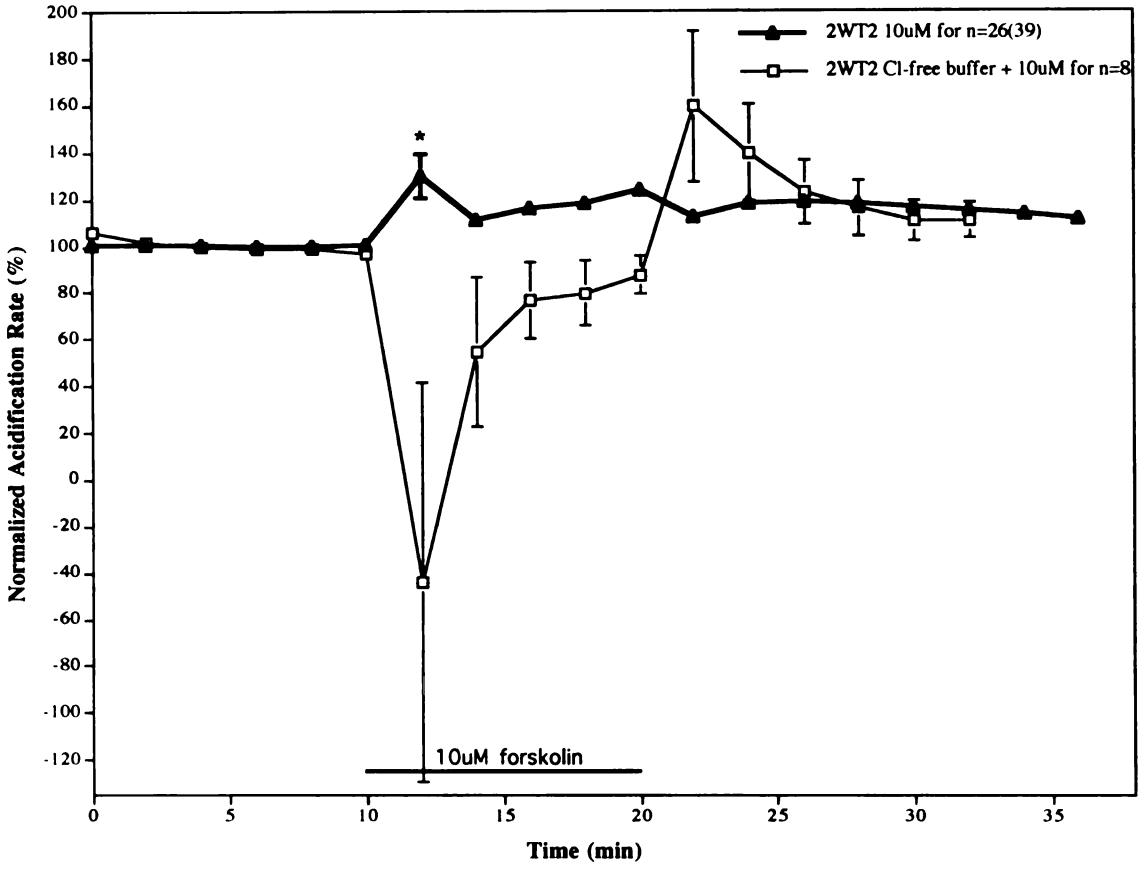

Figure 23. Comparison of normalized acidification rates of cells stably expressing wild type CFTR (2WT2) in normal and Cl^- free media. An exposure to 10 μM forskolin in running media elicited an increase in acidification rate in wild type cells of about 35%. Four minutes following the exposure to forskolin acidification rate in wild type cells was reduced to approximately 25%. In Cl^- free media containing 10 μM forskolin, the acidification rate of wild type cells was rapidly decreased by about 140%. This decrease in acidification rates of wild type cells was short lived but significantly lower than of those in running media. The data suggests continued, and even increased, alkalinization of the extracellular environment in wild type cells despite the Cl^- free media. Each point represents an average of n=8 for Cl^- free buffer, n=26(39) for running media (* $P < 0.05$; error bars as SEM).

DISCUSSION

Cystic fibrosis (CF) is the most common fatal recessive genetic disease effecting caucasians in the United States (Quinton, 1999). About 5% of the caucasian population are asymptomic carriers and about 1 in every 2,500 children born are affected by the disease. CF is caused by the mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The gene is found on the long arm of chromosome seven, and when expressed, CFTR codes for a small, cAMP-stimulated Cl^- channel found on the apical surfaces of epithelial cells (Wine, 1991). This channel facilitates the free movement of salts and water in and out of cells (Quinton, 1999). The mutation of the CFTR gene causes CF and affects many organs in the body such as the skin, lungs, liver, pancreas, small intestine and reproductive tract. Overall, CF is characterized as a disease with defective electrolyte transport and clogging of the epithelial lined ducts and surfaces with thick, sticky mucus. The majority of CF patients, as much as 90%, eventually die of lung disease due to high bacterial infection rates (Welsh and Smith, 1995).

Our previous results indicated that the expression of functional CFTR may significantly alter extracellular pH via control of HCO_3^- efflux (Luckie et al., 2001). Recent reports even suggest that CFTR in fact may be a Cl^-/HCO_3^- exchanger (Choi et al., 2001a). In our current research we looked at four possible models of CFTR in the transport of HCO_3^-: 1) CFTR as a Cl^- channel; 2) CFTR as a Cl^-/HCO_3^- anion exchanger (AE); 3) CFTR as a Cl^- channel and an AE; and 4)

CFTR as a Cl^- channel that also transports HCO_3^- and regulates Cl^-/HCO_3^- exchanger activity. In these studies, I looked at the effect of stimulators and inhibitors of CFTR and AEs' via parallel studies of iodide (anion) efflux and changes in extracellular acidification (pH_o).

The rationale for my study was that recently a group headed by Shmuel Muallem published new findings that have shaken up the field of cystic fibrosis research (Choi et al., 2001a; Choi et al., 2001b). Muallem's group was interested in the secretion of HCO_3^- in CFTR expressing tissues. They studied pancreatic sufficient and insufficient CF mutations by comparing the Cl^- transport and HCO_3^- transport in cells expressing these mutations (Choi et al., 2001a). Their results indicated that some pancreatic insufficient mutations (as found in patients with severe disease symptoms) exhibited normal Cl^- transport, while their HCO_3^- transport and in turn pH regulation were aberrant. This suggested that defective HCO_3^- transport and pH regulation might have an equal if not greater role than chloride transport in the pathophysiology of CF (Choi et al., 2001a). In a later publication, they hypothesized that CFTR may, in fact, also be a Cl^-/HCO_3^- exchanger (Choi et al., 2001b).

1. Specific Aims of the Research

The purpose of the current study was to characterize bicarbonate and chloride transport through CFTR and examine specific tenets of the "Muallem hypothesis," both experimentally and through a literature review, that may imply that CFTR acts as a Cl^-/ HCO_3^- anion exchanger (AE). The specific aims of my

research were:

I. to characterize the Cl^- channel conductance properties of CFTR in selected mammary epithelial cell lines;

II. to examine the conditions necessary for CFTR dependent HCO_3^- conductance;

III. to test the Muallem hypothesis through both my own experiments and through a literature review of studies in other laboratories.

Our study focused on two C127 mouse mammary epithelial cell lines: 2WT2 (expressing wild type CFTR) and ΔF508 (expressing the most common CFTR mutation, deletion of phenylalanine at position 508). They are stable cell lines transfected with the BPV vector carrying wild type or mutant CFTR cDNA. We also used control cells transfected with the BPV vector alone.

In our data collection, we utilized microphysiometer technology to determine the importance of pH and HCO_3^- in CF and to test CFTR as a Cl^-/HCO_3^- exchanger. The microphysiometer can detect subtle changes in extracellular pH (pH_o) and the rate at which a cell acidifies its extracellular environment (McConnell et al., 1992). Cells were perfused with physiological buffers containing various stimulators and inhibitors and the resulting pH_o was monitored (Table 1). Bicarbonate is one of the most important physiological pH buffers, and thus we were able to monitor its transport through transient changes in pH of the extracellular environment. We also used an ORION® iodide electrode system to measure and directly record anion (iodide) flux through the CFTR channel expressed in these mammalian cell lines. These assays were

used to record CFTR channel activity and its response to various inhibitors and stimulators (Table 1).

2. Proposed Models of CFTR to Examine

Results published in the field of cystic fibrosis so far most often point to CFTR being solely a Cl^- channel and the explanation of the pathology of the disease (dehydrated and clogged ducts) as a defect in the transport of Cl^-. As discussed previously, however, there are some results that are not easily explained by a defect in Cl^- transport alone. The lung disease (leading to death in 90% of CF patients), characterized by a buildup of mucus and preferential infection by certain pathogens, is not well understood and has been the hardest consequence of the CFTR mutation to explain solely by abnormal Cl^- transport (Welsh and Smith, 1995; Smith et al., 1996). Therefore, it may be necessary to examine CFTR as a more global regulator of electrolyte transport influencing other anions, such as HCO_3^-, and possibly extracellular pH. In the current research, we attempted to characterize bicarbonate and Cl^- conductance through CFTR. In this study, four specific models of CFTR were tested:

2.1. CFTR as a Cl^- Channel

This model assumes CFTR is simply a Cl^- channel that does not directly influence or regulate any other transporters or transport any other ions (such as bicarbonate). This model is simplistic, but may be all that is relevant to the disease. It has been previously shown that CFTR does conduct HCO_3^- ions

(Poulsen et al., 1994), although the importance and amount of this transport may be insignificant. If this model is a true representation, we would expect to make the following observations:

- Little or no changes in pH_o when CFTR inhibitors (glibenclamide and DPC) or a stimulator (forskolin) are used (Cl^- doesn't directly affect pH); however Cl^- could indirectly affect AE function and thus result in changes in pH_o (use of an AE inhibitor could resolve this question);
- A possible change in a pH_o when AE inhibitor (DIDS) is used (as a result of inhibition of other AE(s) present, for example a Cl^-/HCO_3^- exchanger present on the apical side of epithelial cells); however if change in pH_o is observed it should not be forskolin-dependent;
- No change in pH_o of Cl^- free media following forskolin exposure;
- Decrease in iodide efflux rate with the use of CFTR inhibitors;
- Increase in iodide efflux rate with the use of a CFTR stimulator;
- Iodide efflux rate would remain the same when compared to the control with the use of AE inhibitor (Table 1).

2.2. CFTR as a Cl^-/HCO_3^- Exchanger (AE)

In 2001 a group headed by Shmuel Muallem published new results claiming that CFTR is a Cl^-/HCO_3^- exchanger (Choi et al., 2001b). Thus, one of the possible models to examine (based on Muallem's findings) could be that CFTR is simply an AE (without the Cl^- channel component). Multiple groups have shown that CFTR allows for transport of both Cl^- (Quinton, 1983) and HCO_3^- ions

(Poulsen et al, 1994;) but no evidence thus far was reported supporting this model. If this model is correct we would expect to see:

- Bicarbonate transport through CFTR should be Cl^- dependent and stimulated with forskolin;
- A decrease in acidification rate when glibenclamide and DPC are used (no evidence of inhibition of AE's);
- A decrease in acidification rate when a known CFTR stimulator (forskolin) is used;
- An increase in acidification rate when an AE inhibitor (DIDS) is used;
- Increase in acidification rate in Cl^- free media following forskolin exposure (due to inhibition of HCO_3^- transport);
- There would be no (or very little) iodide efflux rate recorded since iodide transport occurs through a channel (Table 1).

2.3. CFTR as a Cl^- Channel and a Cl^-/HCO_3^- Exchanger

Still keeping in mind Muallem's findings, we also can assume that CFTR has an ability to be both a Cl^- channel as well as a Cl^-/HCO_3^- exchanger. For our purposes we are presuming, though, that either of these CFTR conformations would be "expressed" only one at a time. The focus of the research was to test the ability of CFTR to conduct both Cl^- and HCO_3^- in exchanger fashion using the microphysiometer studies. In this model we predicted that in the case of CFTR being an exchanger and a channel the following should be observed:

- Bicarbonate transport through CFTR should be Cl^- dependent and

stimulated with forskolin;

- Increase in acidification rate in Cl^- free media following forskolin exposure (due to inhibition of HCO_3^- transport);
- Glibenclamide and DPC should stop efflux of chloride through the CFTR channel, but most likely would not prevent action of CFTR as an exchanger (no evidence of such action); thus acidification rate should remain decreased (not affected) as bicarbonate should still leave the cell through an AE;
- DIDS (an AE inhibitor) should not affect bicarbonate movement through CFTR as a channel, it should, however, inhibit bicarbonate movement through its AE; acidification rate in wild type cells should therefore increase since bicarbonate movement through CFTR's AE has been inhibited;
- Iodide efflux rates should be comparable to the first model presented (decrease with CFTR inhibitors; increase with CFTR stimulator; and remain the same as compared to the control with the use of AE inhibitor) (Table 1).

2.4. CFTR as a Cl^- Channel With Regulation of a Cl^-/HCO_3^- Exchanger Activity

The final model of CFTR activity tested assumes that CFTR is primarily a Cl^- channel that through its function and/or structure, affects other exchangers among those that are also Cl^-/HCO_3^- exchangers. In this model it is also assumed that CFTR, even though primarily a Cl^- channel, does allow for passage of other

anions as well (such as bicarbonate). In order to support this model the following would be expected:

- A decrease in acidification rate when CFTR stimulator (forskolin) is used;
- An increase in acidification rate when CFTR inhibitor is used;
- A possible change in pH_o when an AE inhibitor (DIDS) is used (as a result of inhibition of other AE(s) present);
- Decrease in acidification rate of Cl^- free media following forskolin exposure (due to passage of bicarbonate through CFTR);
- Iodide efflux rates should be comparable to the first model presented (decrease with CFTR inhibitors; increase with CFTR stimulator; and remain the same as compared to the control with the use of AE inhibitor) (Table 1).

3. Iodide Efflux Studies

3.1. CFTR Activator Studies

In order to characterize Cl^- conductance properties of CFTR, we first performed iodide efflux studies where cells were treated with a CFTR activator, 10 μM forskolin. Forskolin treatment was used to validate iodide efflux studies as an appropriate method to monitor CFTR activity. As expected, 10 μM forskolin treatment increased iodide efflux in cells expressing wild type CFTR by 3.5 fold (Figure 5). The increase in forskolin-elicited iodide efflux in wild type cells was significantly different than of both mutant and control cell lines ($p<0.01$). Forskolin activates CFTR through an increase in $[cAMP]_i$, hence activating the PKA

pathway and consequent phosphorylation of the R domain of CFTR (Rommens et al., 1991). There was no significant increase in efflux in cells transfected with mutant CFTR, or in control cells. This indicates there is a lack (or severe reduction) of CFTR cell surface expression in these cell lines, since even a small number of CFTR molecules carry a significant amount of iodide and would be detected (Figure 5). Both mutant and control cell lines had a certain level of background noise presumed to be either iodide bound to the cell membrane or iodide leaving cells through other channels. It was assumed that the majority of iodide efflux observed was recorded through Cl^- channels (mainly CFTR) and as AE mechanisms prefer Cl^- over I^-, that the transport through such mechanisms would be insignificant (Venglarik et al., 1990). It has been shown that I^- does not compete with Cl^- with AE mechanisms, further supporting this assumption (Humphreys et al., 1994). These results (the forskolin-elicited increase in iodide efflux) supported all models but model II that assumes CFTR is only an AE, and thus would not allow for transport of iodide.

Mutant and control cell lines had a slow steady increase in iodide efflux throughout the experiment. This was later observed in all iodide efflux experiments for all cell lines. It was found to be comparable between the different cell lines with an average increase in rate (change in concentration over time) of 0.07-0.08. This occurrence could be explained by a potential increase in temperature of media, by lysing cells that are releasing their iodide content, or by existence of other efflux mechanisms.

This set of experiments established that iodide efflux was a reliable method of differentiating between cells expressing wild type CFTR and mutant CFTR, or control cells not expressing CFTR. Iodide efflux was hence used to characterize Cl^- conductance through CFTR. We treated cells with different inhibitors and stimulators of CFTR and AE's and observed their consequent iodide efflux response as an indication of CFTR activity.

3.2. AE Inhibitor Studies

Previously it was shown that CFTR is not affected by DIDS (a general AE inhibitor) (Sheppard and Welsh, 1999). If, however, CFTR contains AE activity, DIDS, an AE inhibitor, should be able to inhibit its function. Therefore we sought to characterize Cl^- conductance through CFTR in cells treated with 500 μM DIDS (AE inhibitor) and 10 μM forskolin. CFTR (a Cl^- channel) was expected to be unaffected. If inhibition was recorded it would indicate an AE component and/or qualities of CFTR.

In wild type cells, simultaneous DIDS and forskolin treatment resulted in similar mean iodide efflux as treatment with forskolin alone. DIDS and forskolin treatment in wild type cells yielded a 6.5 fold increase as compared to the pretreatment state (Figure 9A). The mean increase in efflux rate of wild type cells treated with both DIDS and forskolin was slightly higher (though not significantly different) than the mean increase in efflux of wild type cells treated only with forskolin (Figure 9A). This suggests that forskolin-elicited iodide efflux through

CFTR was not blocked by addition of an AE inhibitor, thus confirming the hypothesis that DIDS in not a CFTR inhibitor.

When comparing the increase from pretreatment to post-treatment state for each drug combination (DIDS and forskolin vs. forskolin alone), they both yielded similar changes in rate of efflux (0.46 and 0.42 respectively). The difference of base response during the pretreatment state (higher base efflux in cells treated with forskolin alone) could be suggested to also indicate the difference between the two treatments during the drug stimulus. Thus the data were re-plotted to compare only those cells studied simultaneously (on the same day) (Figure 9B). These iodide efflux rates tightly paralleled each other, thus differences in the pretreatment state were reduced. The data again supported the idea that DIDS is not a CFTR inhibitor. The difference in base efflux (shown in Figure 9A) during steady state thus appears to be artifactual. The base efflux rates seemed to vary slightly among different cell samples.

Both sets of mutant cells (receiving DIDS and forskolin or only forskolin) exhibited a small steady increase in iodide efflux rate over time, which was found to be statistically different from each other ($p<0.01$ and $p<0.05$ for different time points) (Figure 10). Responses from both treatments, however, paralleled each other and were found to have increased by only two fold in both treatments for the duration of the entire experiment (2.5-5 minutes). This increase in iodide efflux rate was presumed to be either due to media temperature increase during the experiment progression, due to lysing of cells, or to other efflux mechanisms (Figure 10). When compared to wild type cells, mutant cells did not exhibit a

significant increase in efflux (two fold for mutant cells vs. 6.5 fold for wild type cells). Absence of increased iodide efflux following simultaneous DIDS and forskolin treatment suggested the lack of CFTR activation in mutant cells. This was not a surprise, since mutant cells do not express functional, membrane associated CFTR. These data again confirmed the validity of the iodide efflux assay.

We used 500 μM DIDS treatment as per Lee et al. (1999a), which may have resulted in broad range inhibition in these cell lines. DIDS is an AE inhibitor, and it has been shown that it does not affect CFTR when added extracellularly (Sheppard and Welsh, 1999; Reddy and Quinton, 2002). DIDS is also known as a non-specific inhibitor (Wang et al., 2004). My observations of wild type cells treated with DIDS support the hypothesis that it is not an inhibitor of CFTR (no difference was observed when compared to control). Results obtained from mutant cells however, were inconclusive (due to steady efflux increase not found statistically different from wild type cells). The mutant cells should have not had any increase in iodide efflux (due to lack of CFTR expression), so it is possible that such a high dose of DIDS resulted in increased cell lysing and hence increased extracellular iodide concentration.

DIDS studies did, however, suggest that CFTR did not have an AE component thus further supporting results obtained from forskolin studies. Since iodide efflux in these cells, however, occurs mostly through a channel (i.e. CFTR), the studies did not rule out the existence of an AE that is a part of the CFTR or is regulated by it. Thus the observations from these experiments

support all models but model II. Model II (CFTR as an AE) is the only one that does not account for a channel portion of CFTR.

3.3. CFTR Inhibitor Studies

CFTR inhibition studies were performed to further characterize Cl^- conductance through CFTR. Preliminary work showed that the best inhibition was observed with treatment of cells with 400 μM glibenclamide and 100 μM DPC (inhibitor cocktail). This drug combination was then used for further studies. Application of 400 μM glibenclamide, 100 μM DPC and 10 μM forskolin decreased iodide efflux in wild type cells compared to wild type cells receiving forskolin alone (Figure 11). The wild type cells receiving the inhibitor cocktail and forskolin did have a steady increase in efflux starting in the pretreatment state and continuing until half way through treatment state. Comparing increase before and after forskolin treatment showed that the drug caused a maximum 3.5 fold (350%) increase in rate of efflux, while inclusion of glibenclamide and DPC resulted in only a 1.5 fold (50%) increase in rate of efflux. Mean maximum increase for these two different treatments was found to be not significantly different when performing a two-tailed student t-test ($p=0.06$). Different trial number ($n=20$ for forskolin treatment and $n=8$ for inhibitor treatment) required more rigorous statistical testing and could be a plausible explanation for the lack of statistical significance. None-the-less, the difference in the amount of increase rate (350% vs 50%) indicates substantial inhibition of CFTR with glibenclamide and DPC treatment.

The same treatment in mutant cells did not cause any significant change or difference in the overall rate of efflux in either cells treated with forskolin alone or treated with both forskolin and the inhibitor cocktail (Figure 12). There was no significant difference in the effects of the two treatments ($p=0.24$ at $t=3$; $p=0.02$ at $t=3.5$; $p=0.13$ at $t=4$; $p=0.06$ for at $t=4.5$). They both did, however, elicit a steady increase in efflux rate for the duration of the experiment (Figure 12). The efflux rate increase was comparable in both treatments (1.7 fold with forskolin treatment and 2 fold with simultaneous inhibitor and forskolin treatment). This was also comparable to the previously mentioned background drift observed in mutant cells (Figure 7; Figure 10; Figure 12)

If complete CFTR inhibition was accomplished, then inhibitor and stimulator treatment of wild type cells and stimulator treatment of mutant cells should exhibit similar results. However, wild type cells treated with CFTR inhibitors and stimulators did show a significant difference (an increase) when compared to control treatment of mutant cells with forskolin, indicating again partial inhibition of CFTR ($p<0.05$) (Figure 13). This finding was not unexpected since according to current research findings in the literature there are no complete and specific CFTR inhibitors known (Reddy and Quinton, 2002).

Since stock glibenclamide (CFTR inhibitor) was dissolved in DMSO, I also carried out vehicle control studies. Cells were treated with 0.4% DMSO and forskolin and the responses were compared to DMSO treatment alone. In wild type cells, 0.4% DMSO and 10 μM forskolin treatment caused a 5.5 fold increase in iodide efflux rate (Figure 14). The same treatment in mutant cells did not cause

any significant change and efflux remained steady for the duration of the study. It was however significantly different (lower) as compared to wild type cells ($p<0.01$) (Figure 15; Figure 16). DMSO treatment alone did not cause any significant change in iodide efflux rate in either wild type or mutant cell lines (Figure 14; Figure 16).

Looking at forskolin treatment alone, it only caused a 3.5 fold increase in efflux rate of wild type cells, while DMSO and forskolin treatment caused a 5.5 fold increase. This would indicate that DMSO treatment might actually have a stimulatory effect on the efflux rate (possibly due to increased dispersal of forskolin into the cell). Forskolin increases cAMP by directly stimulating the cytoplasmic region of adenylate cyclase (Yoo et al., 2004). Therefore, it needs to be dispersed into the cell for its actions to take affect. If this is the case, the glibenclamide and DPC results (lack of complete CFTR inhibition) might be easier to explain. DMSO could have had an opposite effect on inhibition, thus yielding lower inhibition with glibenclamide and DPC. These data support the hypothesis that the glibenclamide and DPC cocktail is useful as a substantial CFTR inhibitor that could be applied in further studies. Since full inhibition was not recorded and anion flux was still observed, the results do not support model II (CFTR as an AE), while providing support for all others previously presented.

3.4. Conclusions From Ion Flux Studies

Iodide efflux studies showed that cells stably expressing wild type CFTR are stimulated by forskolin (as indicated by an increase in iodide efflux rate). The

AE inhibitor DIDS was not found to have an inhibitory effect on these cells, while glibenclamide and DPC treatment (CFTR inhibitors) were found to only partially inhibit forskolin-elicited iodide efflux. Considering the four models of CFTR function previously presented, only CFTR as an AE was expected to behave distinctly differently in efflux studies. If this was true, we did not expect to see any iodide efflux (as iodide is transported through channels). The other three models (CFTR as a Cl^- channel; CFTR as both a Cl^- channel and an AE; and CFTR as a Cl^- channel with the regulation of an AE) were expected to behave similarly and demonstrate (i) an increase in efflux with forskolin stimulation, (ii) a decrease in efflux with CFTR inhibition and (iii) a comparable efflux between the control and AE inhibitor. Therefore, since all of these expectations were observed, our iodide efflux results do not support the CFTR as an AE model. However, they do not differentiate among the other three models.

4. Microphysiometer Studies

4.1. CFTR Activator Studies

Examination of the conditions necessary for CFTR dependent HCO_3^- conductance were achieved through usage of Microphysiometry, which detects the rate at which a cell acidifies its extracellular environment. When comparing the acidification rates of cells transfected with vector alone (control), and cells stably expressing wild type or mutated CFTR, 10 μM forskolin elicited a smaller increase in acidification rate in wild type cells than in mutant and control cells (Figure 17). An initial approximate increase in acidification rate of 90% (for

mutant cells) and 35% (for wild type and control cells) was observed (Figure 17). Four minutes following the forskolin treatment, acidification rate for mutant cells was still increased by 50% and it remained increased (up to 65%) for the duration of the treatment (Figure 17). Acidification rate of control cells remained increased and continued to rise to 45%, while wild type acidification rate decreased (to a 25% increase compared to pretreatment) and was significantly lower than the other two cell lines for the remainder of the forskolin exposure ($p<0.01$) (Figure 17). Even after the forskolin washout, acidification rate of all three cell lines remained different, although not significantly so.

Forskolin treatment (presumably yielding an increase in $[cAMP]_i$) causes an increase in metabolic activity (proton efflux), thus it would be expected to also elicit an increase in acidification rate of the extracellular environment (Luckie et al., 2001). My results indicate that the increase in acidification rate of the extracellular environment of wild type cells evoked by forskolin is significantly lower than that of mutant and control cell lines. This suggests a molecular mechanism specific to CFTR expressing cells that is able to partially blunt the forskolin-induced acid signal. We also would expect to see no difference in acidification of control cells (not expressing CFTR) and mutant cells (expressing mutant CFTR). Interestingly, our results indicate an increased acidification rate in mutant cells over the control cells. For future experimentation, it would be interesting to investigate the mechanisms of this difference.

The mechanism blunting the acid signal could either be due to decreased proton efflux or increased base efflux (Luckie et al., 2001). Our laboratory has

previously found evidence that this difference is due to increased base (HCO_3^-) efflux through CFTR (Luckie et al., 2001). CFTR's ability to allow for bicarbonate passage, along with Cl^-, and its regulation of Cl^-/HCO_3^- exchangers is also supported by reports in the current literature (Kopelman et al., 1988; Smith and Welsh, 1992; Poulsen et al., 1994; Chan et al., 1996; Poulsen and Machen, 1996; Clarke and Harline, 1998; Lee et al., 1998; Quinton 1999; Illek et al., 1998; Mastrocola et al., 1998; Lee et al., 1999a; Lee et al., 1999b; Ko et al., 2004). The observations indicating forskolin-dependent (i.e. CFTR mediated) bicarbonate flux, do not support expectations for model I (CFTR as only a Cl^- channel). However, they do not differentiate between the other three models proposed. Current research in several laboratories is aimed at trying to determine if this bicarbonate efflux utilizes a Cl^-/HCO_3^- exchanger as its mode of transport.

4.2. AE Inhibitor Studies

A general AE inhibitor DIDS also was used to investigate its ability to inhibit CFTR and therefore to affect pH_o. Treatment with 500 μM DIDS alone in wild type cells resulted in decreased acidification rate by about 25% (Figure 18). When this treatment was combined with 10 μM forskolin, the effect was amplified to a 55% decrease in acidification rate, suggesting still functional HCO_3^- efflux that is consistent with the time course of forskolin treatment (CFTR activation) (Figure 19). These data reinforced the hypothesis that DIDS is not a CFTR inhibitor.

In control cells, DIDS treatment alone elicited a decrease in acidification rate by as much as 150% (Figure 18). On the other hand, when the treatment was combined with forskolin, the acidification rate was decreased only by about 50%, thus allowing for more acid efflux than before (Figure 19). This would indicate that control cells lacked a mechanism that would overcome a rapid proton efflux elicited by forskolin treatment. It would be safe to assume that the difference in the response between wild type and control cells is the result of cell surface expression of CFTR. These results, compared to the wild type response, would indicate a still functional and uninhibited CFTR in wild type cells. DIDS treatment alone in mutant cell lines caused a variable response, however, overall a pattern of decrease in acidification rate was observed (Figure 18).

Since following 10 μM forskolin treatment all three cell lines showed decreased acidification rate in the range of about 50-70%, it is evident that the treatment with 500 μM DIDS did have an effect on an AE common to all three cell lines (Figure 19). It is possible that by inhibiting other anion exchangers present in these cells, DIDS treatment either caused a decrease in proton efflux or increase in base efflux. With forskolin stimulation (and therefore an increase in cell metabolism), however, only CFTR expressing cells were able to even further decrease the acidification rate. The data suggest that base efflux regulated by CFTR was not mediated through an AE, which likely would have been inhibited by DIDS. Rather, the data indicate that base efflux in these cells is most likely mediated through a forskolin-stimulated non-AE pathway, such as CFTR. The data therefore do not support models I (CFTR as a Cl^- channel), II (CFTR as an

AE) or III (CFTR as a Cl^- channel and an AE). Model one was excluded due to observed forskolin-elicited response (indicative of CFTR involvement), while models II and II were excluded due to lack of AE inhibition. It is, however, plausible to assume that model IV (CFTR as a Cl^- channel that allows for transport of bicarbonate and that regulates an AE) is still supported by these observations.

4.3. CFTR Inhibitor Studies

Treatment with 400 μM glibenclamide and 100 μM DPC along with 10 μM forskolin was found to cause a 60-120% increase in acidification rate in all three cell lines (60% for wild type, about 90% for control and 120% for mutant cell lines) (Figure 20). Treatment with 400 μM glibenclamide and 100 μM DPC alone in all three cell lines elicited results similar to a combined treatment with forskolin (45-100% increase in acidification rates) supporting previous iodide efflux results indicating CFTR inhibition with glibenclamide and DPC (Figure 21). There was no statistical difference in the response of any of the three cell lines, thus indicating that inhibition of CFTR in wild type cells increased their resemblance to the control and mutant cells. Considering that forskolin alone also elicited only a 20-25% increase in acidification rates of wild type cells, a 60% (2.4 - three fold) increase as observed with simultaneous inhibitor cocktail treatment is significant and indicative of CFTR inhibition in wild type cells. Glibenclamide and DPC have been used frequently as CFTR inhibitors by other laboratories and have not been found to have an effect on anion exchangers (Reddy and Quinton, 2002). Hence,

the inhibition of CFTR shown here is not likely the inhibition of an AE component. These results further agree with AE inhibitor studies and do not support models II (CFTR as an AE) and III (CFTR as a Cl^- channel and an AE). The results, however, support both models I (CFTR as a Cl^- channel), and IV (CFTR as a Cl^- channel that allows for transport of bicarbonate and that regulates an AE). Even though model I argues that CFTR is only a Cl^- channel, it would be plausible to assume that inhibition of such channel would affect Cl^- concentration, and therefore affect functioning of other cell AEs. This in turn, would affect the pH_o recorded by microphysiometer.

4.4. CFTR Activation in Cl^- Free Media

Our final studies examined the acidification rate of cells in Cl^- free medium with and without 10 μM forskolin. Assuming that CFTR has an AE component, a Cl^- free extracellular environment would create a large concentration gradient for Cl^- to exit the cells and HCO_3^- to therefore enter via an exchanger. Because of this, we were able to examine CFTR as a possible Cl^-/HCO_3^- exchanger.

In our studies, following the switch from Cl^- free media alone, the media containing 10 μM forskolin elicited a decrease by about 140% in the acidification rate of wild type cells, while there was no change in either mutant or control cells (Figure 22). This evidence suggests there is a CFTR-dependent alkalinization of the extracellular environment despite the Cl^- free media, and that the observed decrease in acidification rate and bicarbonate transport through CFTR is not Cl^- dependent. Thus, the data obtained from wild type cells do not support the

involvement of a Cl^-/HCO_3^- exchanger in bicarbonate transport. Although the initial large decrease in acidification rates of wild type cells was short lived, wild type cells sustained a lower acidification rate than mutant and control cells for another pump cycle. Due to variation in data collected, though, the difference between wild type, mutant and control cell lines was not found to be statistically different.

When compared to 10 μM forskolin treatment in running media with Cl^-, 10 μM forskolin in Cl^- free medium was shown to induce even a larger decrease in acidification rate in wild type cells (Figure 23). An exposure to forskolin in media with Cl^- elicited up to a 35% increase in acidification rate, which was found to be significantly different from the 140% decrease observed in Cl^- free media ($p<0.05$) (Figure 23). The increase in forskolin-dependent bicarbonate flux in Cl^- free media (as compared to Cl^- containing media) was unexpected. In order to equilibrate cells to a new medium and stabilize pH, cells were perfused with Cl^- free medium for 28 minutes before introducing forskolin. It is possible that by creating a Cl^- free environment that is maintained extracellularly for long periods of time (hence also creating a huge concentration gradient), the intracellular Cl^- concentration was also greatly reduced. If the concentration of Cl^- was reduced, the competition with HCO_3^- for passage through CFTR also would be reduced. This could be a plausible explanation for a higher HCO_3^- flux and shorter-lived decrease in acidification rate observed for wild type cells in Cl^- free medium.

The data obtained from Cl^- free studies supported only model IV (CFTR as a Cl^- channel with regulation of an AE). Due to a lack of Cl^- dependent

bicarbonate transport, models II (CFTR as an AE) and III (CFTR as a Cl^- channel and an AE) were refuted. Model I (CFTR as only a Cl^- channel) was also not supported. The observed forskolin dependent decrease in acidification indicated that the bicarbonate flux observed was mediated only following CFTR activation. At the same time, Cl^- free media excluded a possible involvement from any other cell-expressed AEs in mediating the bicarbonate efflux observed.

4.5. Conclusions From pH_o Studies

Microphysiometry studies demonstrated that cells stably expressing wild type CFTR have lower acidification rates when stimulated by forskolin than cells stably expressing ΔF508 mutant CFTR and than control cell lines. The AE inhibitor DIDS was found not to have an inhibitory effect on these cells, allowing for further decreased acidification rates in wild type cells when DIDS and forskolin were simultaneously added. Control cell lines also had decreased acidification rates (increased alkalinization) with DIDS treatment, but this affect was reversed by addition of forskolin. The difference in the response between wild type and control cells can be assumed to be related to functional cell surface expression of CFTR in wild type cells. Glibenclamide and DPC treatment (CFTR inhibitors) were found to inhibit CFTR, as evidenced by increased acidification rates when compared to a forskolin treatment. In inhibitor studies, acidification responses of wild type cells were comparable to those of control and mutant cells.

Considering the models of CFTR function previously presented, only the model presenting CFTR as a Cl^- channel that allows for transport of HCO_3^- and regulates an AE was fully supported. This model assumed that in wild type cells we would observe: (i) a decrease in acidification rate when stimulated with forskolin; (ii) an increase in acidification rate upon inhibition with glibenclamide and DPC; (iii) a decrease in acidification rate in Cl^- free media; and (iv) a decrease in acidification rate upon stimulation with an AE inhibitor (DIDS). As explained above, all of these predictions were supported by microphysiometry studies. It should be noted that DIDS treatment however, was hard to differentiate in any model due to its potential influence on other cell AE's.

The other three models (CFTR as a Cl^- channel; CFTR as both a Cl^- channel and an AE; and CFTR as an AE) were not fully supported. CFTR models as an AE and as both Cl^- channel and an AE assumed that bicarbonate transport would not only be forskolin elicited, but also Cl^- dependent. As shown by the continued decreased acidification rate in Cl^- free media, that was not the case. Also, it was expected that DIDS would inhibit the decreased acidification rate and that glibenclamide and DPC would not, but neither was observed.

Lastly, the model presenting CFTR as only a Cl^- channel assumed that there would be little or no change in pH_o with most treatments. It was expected that there would be no much change in pH_o upon stimulation with forskolin alone. Forskolin is a CFTR stimulator and response elicited by such treatment would indicate a CFTR dependent bicarbonate flux. Even though Cl^- does not directly affect pH, it was predicted that the treatment potentially inhibiting other cell AE's

(DIDS) would elicit a response. We would not, however, expect that the response observed would be further enhanced by forskolin stimulation, as it was evident from our studies. Glibenclamide and DPC treatment resulting in inhibition of CFTR was the only microphysiometer study where the results obtained could support model I. In these experiments the increased acidification rate was recorded. It is presumed that inhibition of CFTR (as a Cl^- channel) would affect Cl^- concentration and thus functioning of other AEs expressed by cell lines used. This in turn, could affect pH_o. Overall, our microphysiometer data support only a model where CFTR is a Cl^- channel that allows for HCO_3^- transport and has a regulatory influence on an AE.

5. Literature Research: A Channel or An Exchanger?

Our studies attempted to clarify the role of CFTR in the transport of HCO_3^- and its influence on extracellular pH. Most of the research published to date has focused on CFTR as a Cl^- channel. Recently, there has been increased interest in CFTR as a global regulator of other channels and transporters, and even as an ion exchanger. Here I present a literature review in the areas so far most examined by the field looking at: 1) CFTR as a Cl^- channel, and 2) its relationship with Cl^-/HCO_3^- exchange.

5.1. Channel Story

Though at the time not referred to as cystic fibrosis, written records and indications of CF date to as early as the 1600's in diaries and folk stories in

Europe (Busch, 1990). These records talk about "bewitched" children with salty foreheads, children presumably affected by CF (Busch, 1990). It was not until later that this characteristic was attributed to cystic fibrosis. The cause of higher salt content in sweat was first described in 1983 (Quinton, 1983).

The first indication that CF was caused by a defect in Cl^- movement across the membrane came in the 1980's. Prior to the 1983 research published by Paul Quinton, the physiological explanation of the disorder was unknown. Quinton carried out a study on isolated sweat ducts from control subjects and from CF patients. These ducts were microperfused and transepithelial potential differences during the microperfusions were monitored (Quinton, 1983). Quinton observed a significantly increased negative potential in the cystic fibrosis sweat duct as compared to the controls. He assumed that the differences recorded were due to differential anion permeability in the CF and control sweat ducts. He concluded that low Cl^- permeability in CF ducts resulted in poor reabsorption of NaCl and hence lead to high NaCl concentration in the sweat of CF patients (Quinton, 1983). It was not until six years later in 1989, that the underlying molecular basis for CF was discovered.

Collaborative efforts of two groups yielded the successful cloning of the CFTR gene (Riordan et al., 1989). The first cDNA clone was isolated from the cultured epithelial cells of the sweat glands of an individual not affected with CF (Riordan et al., 1989). Following cloning of the wild type CFTR, these groups compared the cDNA sequences from the CF and unaffected individuals and isolated two mutant clones from sweat glands of CF affected individuals. Both of

these clones were discovered to have a three nucleotide sequence deletion (loss of phenylalanine at position 508), a mutation that was later attributed to about 70% of the cases of CF. Analysis of the cloned sequences revealed that they coded for a polypeptide of 1480 amino acids with a molecular mass of 168,138 daltons (Riordan et al., 1989). They also concluded that this protein had two membrane spanning domains and two sequences resembling nucleotide (ATP) binding domains (Riordan et al., 1989). Based on these structural features and resemblance to other membrane proteins, defects in the protein (termed CFTR) were concluded to be directly responsible for CF (Riordan et al., 1989). Due to the previous indications of a Cl^- permeability defect in CF, it also was concluded that CFTR was involved in regulation of ion conductance across the apical membrane of epithelial cells. Even though the researchers speculated that CFTR could be a Cl^- channel itself, there was no direct support for that prediction (Riordan et al., 1989). The isolation of CFTR cDNA pushed the CF field ahead and enabled further characterization of CFTR as a channel.

The first step towards characterizing the function of the now isolated CFTR was the creation of cell expression systems. In fact, these expression systems are still being used in CF research today. In the early 1990's, CFTR cDNA was expressed in multiple cell lines. Anderson et al. (1991a) published one of the first reports of CFTR expression and characterization. In an effort to characterize CFTR function, they expressed CFTR cDNA in HeLa, Chinese hamster ovary (CHO), and NIH 3T3 fibroblast cells with the vaccinia-T7 hybrid expression system (Anderson et al., 1991a). They then assessed its anion

permeability with a fluorescence microscopic assay and whole-cell patch-clamp. Following cAMP treatment, an increase in anion permeability and chloride current was recorded in CFTR expressing cells (Anderson et al., 1991a). Based on these results, they concluded that CFTR is most likely a chloride channel itself, thus further supporting the previous prediction of CFTR function.

Later that year, the same research group further supported their prediction of CFTR being a channel. Trying to distinguish whether CFTR was either chloride channel or a chloride channel regulator, they used T84 epithelial cells expressing wild type and mutant CFTR and compared the properties of cAMP regulated currents (Anderson et al., 1991b.). To accomplish this, they mutated several important amino acids in areas thought to form the pore of the channel from charged residues to negative ones, thereby changing the ion selectivity of the pore (Anderson et al., 1991b.). The CFTR ion selectivity prior to these mutations was $Br^- \geq Cl^- > I^- > F^-$, and following the mutations was changed to $I^- > Br^- > Cl^- > F^-$ (Anderson et al., 1991b). They concluded from these data that CFTR was a Cl^- channel itself and not a channel regulator. Even though the previous results of multiple groups had shown that low-conductance Cl^- currents were generated with the expression of CFTR, this was one of the first studies to actually demonstrate that CFTR was a Cl^- channel rather than a regulator (Tabcharani et al, 1992).

The hypothesis that the CFTR functions as a Cl^- channel was even further supported with purification of CFTR and its insertion in artificial membranes (Bear et al., 1992). This group purified a recombinant CFTR from a Sf9 insect cell line

expressing the CFTR and reconstituted it into proteoliposomes (Bear et al., 1992). Following the formation of these vesicles, fusion with a lipid bilayer was accomplished and Cl^- conductance was monitored. The activity observed (chloride currents with properties identical to those found in CFTR expressing tissues) was strong evidence that CFTR is indeed a low-conductance Cl^- channel (Bear et al., 1992).

Even though the evidence that CFTR was a Cl^- channel was strong at this point, soon after the channel function was again being questioned. There were indications that in CF patients other transport functions may have been affected. It was noted that in CF, outwardly rectifying Cl^- channels were defective, and these, typically show higher Cl^- conductance (Egan et al., 1992). These results did not correspond with already supported claims of CFTR being a low-conductance Cl^- channel. Egan et al. (1992) expressed recombinant CF genes in CF bronchial epithelial cells and noted rectification of defective Cl^- secretion that resembled that of CFTR's low-conductance chloride current (Egan et al., 1992). They also demonstrated that the activation of outwardly rectifying Cl^- channels was restored (Egan et al., 1992). The evidence again pointed to CFTR as a Cl^- channel but one that may also regulate other cellular functions such as outwardly rectifying Cl^- channels.

5.2. Exchanger Story

Following identification of the CFTR gene, it became clear that CFTR and its function might be more complex than previously assumed. Since then, the

function of CFTR as a chloride channel has been confirmed by many different research groups, but it also was shown that CFTR might have a much more complex role in electrolyte transport and balance. The control and regulation of CFTR over the transport of other molecules has since been suggested, among others, that of bicarbonate. Recently there has been some indication that impaired transport of bicarbonate through CFTR might be even more important to development of CF than that of defective chloride transport alone, and that CFTR might even be a Cl^-/HCO_3^- exchanger (Choi et al., 2001a; Choi et al., 2001b). The evidence for this claim was, however, somewhat weak.

Simultaneously with the beginning of our study in 2001, Shmuel Muallem and colleagues published results of a study dealing with various pancreatic sufficient and insufficient mutations of CF. The focus of the majority of their previous research was on impaired HCO_3^- secretion in CFTR expressing tissues. Previously, they worked on both, HCO_3^- secretion as well as salvage mechanisms, and these studies indicated that CFTR was involved in both (Lee et al., 1999a; Lee et al., 1999b; Ahn et al., 2001). They used HEK293 cells transiently transfected to express wild type and various mutations of CFTR. These cells were then used for studies monitoring their intracellular pH (pH_i) with BCECF dye, as well as Cl^- channel flux with MQAE dye. Measurements of $[Cl^-]_i$ with MQAE dye were shown to correspond to the measurement of Cl^- current. The cells were stimulated with 5 μM forskolin to stimulate CFTR activation and Cl^- free media was used in pH studies. Their results indicated that some pancreatic insufficient mutations had normal Cl^- transport while their pH

regulation, and in turn HCO_3^- transport, were defective (Choi et al., 2001a). They indicated that defective HCO_3^- transport and pH regulation might have an equal if not greater role compared to impaired chloride transport in the pathophysiology of CF. In a follow-up paper, the authors hypothesized that CFTR may, in fact, be a Cl^-/ HCO_3^- exchanger (Choi et al., 2001b). The conductance of HCO_3^- through CFTR and pH regulation disruption in CF (resulting in the acidification of the environment of the CF lung) has long been known (Smith and Welsh, 1992; Poulsen et al., 1994; Coakley et al., 2001; Luckie et al., 2001; Tate et al., 2002; Coakley et al., 2003). This was the first time a group in a field of CF research has made such a dramatic exclamation about the importance and mode of bicarbonate transport.

Following publications of these results, much CF research has been focused on the importance of bicarbonate transport and pH regulation. The data and claims published since, even by this group, have been more indicative of regulation and communication of several members of the SLC26 family of chloride-bicarbonate exchangers with CFTR (Ko et al., 2002). In 2002, Muallem and colleagues published data showing that the molecular mechanism for aberrant and yet CFTR-dependent bicarbonate transport associated with CF, is due to regulation of members of the SLC26 family by CFTR (Ko et al., 2002). Their results indicated that CFTR increases activation of Cl^- and HO^-/ HCO_3^- transport by three members of this family: DRA, SLC26A6 and pendrin (Ko et al., 2002). DRA was also indicated in the interaction with CFTR in mouse pancreas

and was found to have altered function when certain CFTR mutations were present (R117H and G551D) (Ko et al., 2002).

Interaction and regulation of a HCO_3^- salvage mechanism via Na^+-HCO_3^- cotransporter 3 (NBC3) with CFTR also was shown by this group, again supporting the regulation of HCO_3^- transport by CFTR, but through interactive mechanisms rather than direct involvement (Park et al., 2002). In these studies, HEK cells were stably and transiently transfected with NBC3. The authors looked at bicarbonate transport and characterized it using various inhibitors and activators. The results indicated that CFTR and NBC3 collaborate in the transport of HCO_3^- and maintain a protein-protein interaction through their respective PDZ domains (Park et al., 2002). This interaction was obligatory for regulation of NBC3 and the HCO_3^- salvage mechanism by CFTR (Park et al., 2002).

Early in 2004, this group published two new papers further addressing the question of bicarbonate transport through CFTR and its role in regulation of Cl^- /HCO_3^- exchange. Ko et al. (2004) showed direct molecular interactions between CFTR and members of the SLC26 family of Cl^-/HCO_3^- exchangers. They focused their research on two members of this family, A3 (DRA) and A6. They found a reciprocal regulation between these two Cl^-/HCO_3^- exchangers and CFTR. SLC26 transporter activity was increased when CFTR was activated by phosphorylation (Ko et al., 2004). Interestingly, they also found that CFTR channel open probability was increased when SLC26 transporters were present (Ko et al., 2004). The interaction of these proteins was maintained through their attachment to PDZ binding proteins. Both CFTR and SLC26 transporters contain PDZ

binding domains and are found to bind to similar PDZ scaffold proteins such as EBP50 and EKARP (Ko et al., 2004; Gray, 2004). When they attach to PDZ binding proteins and are in proximity, the phosphorylation of the R domain of CFTR causes its binding to a conserved STAS domain of SLC26 transporters (Ko et al., 2004). Ko and colleagues found that this molecular interaction is responsible for reciprocal upregulation of CFTR and SLC26 transporters and has a significant impact on regulation of chloride and bicarbonate transport in epithelial cells. They also have further proposed a mechanism for differential bicarbonate secretion in the pancreatic duct system which depends on the expression of SLC26 transporters.

Shcheynikov et al. (2004) examined control of Cl^-/ HCO_3^- transport by measuring membrane potential and pH_i and/or current in *Xenopus oocytes* expressing CFTR. Their results indicated a dynamic control of the Cl^-/ HCO_3^- permeability ratio of CFTR that was dependent on external Cl^- concentration. When Cl^- concentration was low or media was Cl^- free, their results pointed to OH^- and HCO_3^- influx but no HCO_3^- efflux. They concluded that permeability of CFTR to HCO_3^- at Cl^- free times is low. They hypothesized that CFTR exists in two conformations (a Cl^- only and a Cl^- and OH^-/ HCO_3^- permeable state) and that the switch between these two states is the external Cl^- concentration. However, according to them, most of HCO_3^- secretion is mediated by the SLC26 transporters, as discussed above, and the switch between the two conformation of CFTR channel serves as a "valve to clamp the HCO_3^- gradient across the luminal membrane according to the Cl^- gradient" (Shcheynikov et al., 2004). They

suggested that this effect may be important in tissues that secret fluid with high [HCO_3^-], such as the pancreas and salivary glands (Shcheynikov et al., 2004). The final conclusion of this group seems to be that CFTR is a channel with two conformational states (a Cl^- only and a Cl^- and OH^-/HCO_3^-permeable state), that also controls Cl^-/HCO_3^- exchange through a PDZ domain mediated interaction with members of the SLC26 transporter family.

5.3. Conclusions From Literature Review

Historically, work on CF mechanisms has mostly focused on the first model of CFTR function (CFTR as a Cl^- channel). Since 1983, when the Cl^- defect was first characterized, there has been much work carried out supporting CFTR's role as a channel. Recently, more interest has been directed toward looking at CFTR's relationships with other cell transporters. As presented in the literature review above, CFTR was found to be in close regulatory interaction with other cell transporters and AE's. These data clearly support the fourth model which suggests that CFTR is a Cl^- channel that allows for HCO_3^- transport and regulates one or more AEs. There have been no results published in the literature which indicate that CFTR is only an AE, and only one study (Choi et al., 2001b) stating that CFTR has an AE component. This study (Choi et al., 2001b) seems to have since been refuted (Shcheynikov et al., 2004; Ko et al., 2004). Thus, based on the current literature review, the field remains divided on the issue of CFTR and its role in regulation of HCO_3^- transport and pH. In my opinion, data reported thus far could support both models I (CFTR as a Cl^- channel only)

and IV (CFTR as a Cl^- channel with regulation of an AE). However, if model one is favored, the discrepancies in experimental data still need to be accounted for.

6. Hypothesized Model of CFTR Function Based on All Current Experimental Data

At the beginning of this project, I set out to characterize regulation of Cl^-/HCO_3^- transport and extracellular pH by CFTR. More specifically, I wanted to distinguish Cl^-/HCO_3^- transport through CFTR as either channel mediated (mostly supported by the field) or exchanger mediated (as proposed by Choi et al., 2001b). I have used two different sets of studies, iodide efflux and microphysiometer experiments, to examine the exchanger hypothesis. Iodide efflux results were used to characterize Cl^- conductance through CFTR and examine the effectiveness of inhibitors and stimulators used. Microphysiometer studies were used in examining the exchanger hypothesis. Examining this question, I set up four possible models of CFTR function:

I. CFTR as a Cl^- channel
II. CFTR as an AE
III. CFTR as both a Cl^- channel and an AE
IV. CFTR as a Cl^- channel that allows for transport of HCO_3^- and regulates an AE

Previously, I have defined three characteristics expected if CFTR was an exchanger: 1) bicarbonate transport should be Cl^- dependent and stimulated with forskolin, 2) glibenclamide and DPC should stop efflux of chloride through the

channel, but should not prevent action of CFTR as an anion exchanger, and 3) DIDS should block bicarbonate movement thorough CFTR's anion exchanger in expression cells. None of these characteristics were observed in my data.

Glibenclamide and DPC were found to prevent the forskolin stimulated bicarbonate efflux through CFTR (as observed by increased acidification rate following the inhibitor treatment). DIDS treatment did not prevent bicarbonate efflux stimulated with forskolin; rather it was found to increase it in all cell lines used. This was indicative of inhibition of AEs found in all three cell lines, and not an AE specific to the CFTR expressing line. In addition, the decrease in acidification rate during the simultaneous DIDS and forskolin treatment was greater than with DIDS alone only in wild type cells. These results indicated that upon CFTR activation with forskolin there is an additional base efflux to compensate for increased metabolic rate.

Finally, the most striking piece of evidence came from studies with Cl^- free media where forskolin treatment in wild type cells caused an even larger decrease in acidification than it did in Cl^- containing media. HCO_3^- transport observed through CFTR was not Cl^- dependent and therefore was most likely not mediated by an exchanger mechanism.

Similarly, as shown by the data recorded in the field since the start of my Masters study, the results of Choi et al. (2001b) that were indicative of CFTR serving as a Cl^-/ HCO_3^- exchanger, could be explained with a very tight interaction of CFTR and other Cl^-/ HCO_3^- exchangers, such as members of SLC26 transporter family like DRA and A6. CFTR has been shown to regulate

these transporters and therefore Cl^-/ HCO_3^- exchange through interactions with PDZ domains (Ko et al., 2004). PDZ mediated interactions of CFTR with other transporters, however, are not an isolated event with SLC26 family. CFTR also has been shown to interact with multiple PDZ proteins such as EBP50, Na^+/H^+ exchanger type 3 kinase A, CAP70, and so on (Kunzelmann, 2001). It has been suggested as a factor in regulation of epithelial fluid and electrolyte transport that not only functions as a Cl^- channel but also affects other exchangers and channels (Lee et al., 1999b; Ahn et al., 2001). It is thought that CFTR interacts with other cell proteins, such as ENaC, through their respective PDZ domains and scaffolding proteins (Kunzelmann, 2001). CFTR has been suggested to affect Na^+ absorption through epithelial Na^+ channels (ENaC), K^+ secretion through luminal K^+ channels, and HCO_3^- salvage through Na^+/HCO_3^- exchange (Lee et al., 1999b; Ahn et al., 2001). These studies all support CFTR function as presented by model IV (CFTR as a Cl^- channel that also allows for transport of HCO_3^- and regulates an AE).

It is important to note that our predicted "models" are just a beginning to answering the question of CFTR function. For example, we have not accounted for a model assuming that CFTR is mainly a Cl^- channel that also allows for a bicarbonate transport but does not regulate other transporters. This model is very similar to model IV presented in this work (CFTR as a Cl^- channel that also allows for transport of HCO_3^- and regulates an AE). Essentially, a Cl^- channel that allows for bicarbonate transport would at times be same as model IV, the only difference would be observed when CFTR is involved in regulation of other

transporters and/or exchangers. Even though our research did not show the interaction of CFTR with other proteins (as it is assumed in model IV) there has been much evidence in recent literature of such occurrences.

The exact influence of CFTR on these transporters and its molecular mechanism of action are still not completely characterized. Yet it seems likely that the field of CF research may be heading towards a better understanding of all of these interactions and their importance in development of the disease. It is my opinion, that the results from the current research and those of others support the idea that CFTR is a Cl^- channel that allows for HCO_3^- transport and regulates Cl^-/ HCO_3^- exchange (model IV). My results specifically do not support the models I (CFTR as only a Cl^- channel), II (CFTR as an AE) and III (CFTR as a Cl^- channel and a Cl^-/ HCO_3^- exchanger).

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