UNIVERSITY OF ALBERTA

Ion Transport in Human Airway Epithelium: The Role of Basolateral Chloride Channels

By

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Dedicated to my family: my parents Mircea and Georgeta,
my wife Florentina and my son Nicholas

Abstract

The first objective of my project was to identify Cl^- channels at the molecular level in the basolateral membrane of airway epithelial cells. We focused on a new family of Cl^- channels, bestrophins, which have been previously identified in retinal pigment epithelium. RT-PCR, Western blot and immunohistochemistry studies revealed the presence of bestrophin in airway epithelial cells. Decreasing bestrophin expression using siRNA resulted in diminished $^{36}Cl^-$ flux across the basolateral membrane. These studies also showed that bestrophin regulation is similar to that of endogenous basolateral Cl^- channels. The data indicate that bestrophin contributes to the basolateral membrane conductance in airway epithelial cells.

The existence of basolateral Cl^- channels in airway epithelium has been reported in several studies but little is known about their role in the regulation of anion secretion. Therefore, my second objective was to characterize the regulation of these channels by nitric oxide (NO) in the epithelial cell line Calu-3. Transepithelial measurements showed that NO donors activated a basolateral Cl^- conductance sensitive to 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and anthracene-9-carboxylic acid (9-AC). Apical membrane permeabilization studies confirmed the basolateral localization of NO-activated Cl^- channels. Experiments using 8Br-cGMP and selective inhibitors of soluble guanylate cyclase and inducible NO synthase (ODQ and 1400W, respectively) demonstrated that NO

activates Cl^- channels via a cGMP-dependent pathway. Anion replacement and $^{36}Cl^-$ flux studies showed that NO affects both Cl^- and HCO_3^- secretion. Currently, two different types of Cl^- channels are known to be present in the basolateral membrane of epithelial cells: Zn^{2+}-sensitive CLC-2 and DIDS-sensitive bestrophin channels. S-nitroso-glutathione (GSNO) activated Cl^- conductance in the presence of Zn^{2+} ions, indicating that CLC-2 channel function was not affected by GSNO. In contrast, DIDS completely inhibited GSNO-activated Cl^- conductance. Bestrophin immunoprecipitation studies showed that under control conditions bestrophin was not phosphorylated, but it became phosphorylated after GSNO treatment. I conclude that bestrophin is likely to be a key player in basolateral Cl^- conductance and play a major role in NO-dependent regulation of anion secretion in Calu-3 cells.

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List of abbreviations

ABC	ATP-Binding Cassette
9-AC	Anthracene-9-carboxylic Acid
AMD	Age-related Macular Dystrophy
ASL	Airway Surface Liquid
BK	Big Conductance Ca^{2+} dependent K^+ channel
CAMKII	Ca^{2+}/calmodulin-dependent Protein Kinase II
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CLC	Voltage Gated Cl^- Channel
CLCA	Ca^{2+}- Dependent Cl^- Channel
DIDS	4, 4'-Diisothiocyanatostilbene-2, 2'-Disulfonic Acid
DPC	Diphenylamine-2-carboxylic Acid
EDRF	Endothelial-Derived Relaxing Factor
ENaC	Epithelial Na^+ Channel
GSNO	S-Nitrosoglutathione
HDL	High Density Lipoproteins
HEK293	Human Embryonic Kidney Epithelial Cells
HMG CoA	Hydroxymethylglutaryl-CoA
IK	Intermediate Conducatance Ca^{2+} Dependent K^+ Channel
I_{CIN}	Swelling-Activated Chloride Channel
Isc	Short-Circuit Current

J_{AB}	Apical to Basolateral Flux
J_{BA}	Basolateral to Apical Flux
KHS	Krebs-Henseleit Solution
Kir	Inwardly Rectifying K^+ Channel
Kv	Voltage Dependent K^+ Channel
LDL	Low Density Lipoprotein
MDCK	Madin-Derby Canine Kidney Epithelial Cells
NBC	Na^+/HCO_3^- Cotransporter
NBD	Nucleotide Binding Domain
NKCC	$Na^+/ K^+/ Cl^-$ Cotransporter
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPPB	5 Nitro-2-(3-phenylpropilamino) Benzoic Acid
ODQ	1H-[1, 2, 4] oxadiazolol-[4, 3-a] quinoxalin-1-one
ORCC	Outwardly Rectifying Chloride Channel
ORDIC	Outwardly Rectifying Depolarization Induced Cl^- Channel
PDE	Phosphodiesterase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
RPE	Retinal Pigment Epithelium
R_T	Transepithelial Resistance
SK	Small Conductance Ca^{2+} Dependent K^+ Channel

sGC	Soluble Guanylate Cyclase
TMD	Transmembrane Domain
VMD	Vitelliform Macular Dystrophy
V_T	Transepithelial Potential Difference

CHAPTER 1

INTRODUCTION

1. Epithelial ion transport

1.1 Ion transport in epithelial cells

Epithelium consists of layers of cells that cover body surfaces or line tubular organs, protecting from direct contact with external factors. In addition to barrier function, epithelium can transport ions or metabolites from the mucosal side (lumen) to the serosal side (blood) and vice versa. Ion secretion or absorption is achieved by using different combinations of ion channels and transporters.

Epithelial cells are polarized, so membranes with different features face either the mucosal side (apical membrane) or the serosal side (basolateral membrane). An example of the different structure of the two types of membrane is the contrasting distribution of ion channels - in the apical membrane the most important are Na^+ and Cl^- channels, while in the basolateral membrane K^+ channels are prominent.

This polarity is maintained by junctional complexes, made of a network of proteins which contain occludin (85) and claudin (84). The main roles of the junctions are to maintain polarity and to hinder paracellular transport. The transepithelial resistance is a measure of the tightness of the epithelium, the tighter epithelium having a higher resistance. Accordingly, different epithelia can be "leaky", such as epithelia of the gallbladder or small intestine, or "tight", like the epithelia of colon or distal nephron.

Ion channels are composed of integral transmembrane proteins that allow passage of certain ions at low free energy levels. The chemical/structural properties of the channel's pore determine ion channel selectivity.

The majority of ion channels are regulated by gating, which refers to different conformational changes of channel proteins. Ion channel protein could be in the open state (allows ion passage), closed state (does not allow ion passage) or inactive state (channel will open only in the presence of an activator). The tendency of a channel to be open is called open probability (P_o), which is in a range from 0 (closed channel) to 1 (open channel). P_o of various channels is controlled by different factors such as voltage (voltage-dependent ion channels), ligands (ligand-gated ion channels), protein modifications (phosphorylation/nitrosylation) or pharmacological means.

All biological systems are continuously moving towards a state of equilibrium, where there is a lack of impetus for further changes. When a membrane separating two solutions is permeable only to specific ions, these ions tend to flow to the solution with lower concentration and to break the electrical equilibrium across the membrane. The equilibrium potential is the electrical potential required to balance the energy in the chemical gradient and could be calculated using the Nernst equation:

$$E_{ion} = -\frac{RT}{zF}\ln\frac{C_{in}}{C_{out}}$$, where

E_{ion} = electrical potential (mV)

R = natural gas constant

T = the absolute temperature (°K)

z = the valence of the ion

F = the Faraday constant (96500 Coulombs/mol)

C_{in} = the concentration of ion inside the cell (mmol/l)

C_{out} = the concentration of the ion outside the cell (mmol/l)

In the open state there is net flow of charge, which is called current. The current is determined by the membrane potential between the recording electrodes and by the membrane conductance. Current-voltage curve (I-V plot) is used to show specific conductance of different ion channels, which could be linear, inward or outward rectifying. All these parameters can be calculated by using a fundamental rule of electricity, called Ohm's law:

$$G = \frac{I}{E_m - E_{ion}}, \text{ and } \quad R = \frac{1}{G}, \text{ where}$$

E_m - E_{ion} = driving force, expressed by the difference between membrane potential, E_m, and the equilibrium potential, E_{ion}, determined from the Nernst equation.

I = current (amperes, A)

G = conductance (siemens, S)

R = resistance (ohms, Ω)

There are different techniques used to measure ion currents, such as patch clamp, used mainly to assess activity of ion channels in single cells, and transepithelial measurements in Ussing chambers, which measure vectorial ion transport across epithelium.

Patch clamp has been developed by Erwin Neher and Bert Sakmann at the beginning of 1980's in Göttinggen, and for this major discovery they were awarded the Nobel Prize in Physiology and Medicine in 1991 (205). This technique is a valuable tool in studying ion currents through single channels, being able to detect currents in the order of pico amperes.

There are four main configurations that can be used in patch clamping:

- cell-attached patch
- inside-out patch
- outside-out patch
- whole cell patch

In cell-attached, inside-out and outside-out modes few channels or only a single channel protein activity can be measured. These configurations are also useful for measuring macroscopic currents under appropiate conditions. Cell-attached mode can be attained after forming of giga-seal between the pipette and the cell membrane. The giga-seal formation is a critical step of the experiment, since it eliminates most of the electrical noise that interferes with recordings of such low currents (106). The advantage of recording single channel activity in cell attached mode comes from the fact that cell membrane integrity is maintained, and consequently all regulatory mechanisms of ion channel function, such as second messengers and protein kinases, are not disturbed. Among drawbacks of this configuration is the inability to measure the cell resting potential and difficulties in modifying the pipette solution.

The inside-out configuration can be obtained by withdrawing the pipette after a cell-attached mode has been established. The outside-out patch can be done with a similar manoeuvre, but after a whole-cell configuration has been set. In these two configurations, the potential of the patch is equal to the pipette potential (in inside-out Vm = -Vp and in outside-out Vm = Vp); therefore it can be determined accurately. The solutions from the bath and pipette can be easily manipulated as well. However, the major disadvantage is

that what is measured cannot be correlated with the physiological events that occur inside the cell.

In the whole cell mode, after attaining cell-attached configuration, a brief suction is applied inside the electrode, so that the patch membrane is ruptured. In this mode whole cell currents can be measured. Although the whole-cell mode is one of the most frequently used configurations, due to its simplicity and fast analysis, one of its limitations is the possibility of losing regulatory intracellular molecules, such as Ca^{2+} or cAMP, which can diffuse through the patch pipette.

Ion transport across epithelium is important, since it contributes to different physiological functions such as regulating pH, cell volume, secretion or absorption. Depending on the localization in the organism and the main function of a specific epithelium, there are different patterns of the distribution of ion channels and transporters.

One of the main methods to study transepithelial transport is to measure the short-circuit current (I_{sc}) in Ussing chambers. A positive I_{sc} is defined as the anion secretion or the flow of anions from the basolateral side to the apical side. Any epithelial tissue or cell culture that forms highly resistant monolayers can be mounted in Ussing chambers, where it is voltage-clamped at zero and then transepithelial current is recorded. Hans Ussing, who developed this technique in the 1950s, in his quest to understand active Na^+ flux (Na^+/K^+ pump), used radioactively labeled Na^+ to show that the I_{sc} measured across frog skin epithelium was due to sodium transport (295).

This technique was used to study the function of apical chloride channels in airway and intestinal epithelia (6), or the regulation of epithelial Na^+ channels (ENaC)

(174). Although a very powerful technique, sometimes it is difficult to distinguish between the function of apical or basolateral membrane ion channels or transporters (157). One way to circumvent this disadvantage is to use specific blockers or ion substitution in the solutions bathing the apical or basolateral compartments. Another approach is to selectively permeabilize apical or basolateral membranes with antibiotics, such as nystatin or amphotericin B, which form ion selective pores. However, there is a lack of specific inhibitors and the only hope is that most of the ion channels and transporters will be identified at the molecular level, so that specific drugs for different ion channels will be designed.

Retinal pigment epithelium (RPE) is involved in ion and water transport and acts as any other epithelium in the human body in terms of ion secretion or absorption (188;189). It can absorb or secrete water depending on the physiological functions (adaptation to light or dark) (87). One particular feature of this epithelium is the presence of chloride channels on both apical and basolateral membranes. A number of chloride channels, such as CLC-2 (308), CFTR (308;317) and bestrophin (111;220) were identified to be present and functionally important in water secretion/absorption in this epithelium.

Another epithelium with chloride channels on the apical and basolateral membranes is the intestinal epithelium (217). The most important ion channel in the apical membrane is CFTR (193), and lack of expression or malfunction of this channel is responsible for the pathological findings in cystic fibrosis (CF). Recently it was demonstrated that CLC-2 is located on the basolateral membrane of intestinal epithelium.

This channel plays a role in fluid absorption, but its disruption does not exacerbate CF phenotype in CFTR knock-out mice (42;324).

1.2 Airway epithelium

Airway epithelial cells line human airways and act as a barrier, protecting the underlying tissues, such as smooth muscle and nerve terminations, from potential harmful external stimuli. Airway epithelium responds to different stimuli by secreting active mediators, such as cytokines, lipid mediators or reactive oxygen species. It also modifies airway secretion, ion transport and mucociliary clearance when stimulated by airway irritants.

The epithelium of the small airways is cuboidal, with a paucity of mucus producing cells or glands. Alveoli are covered in epithelium, consisting of two types of cells: type II pneumocytes, covering 1-5% of the alveolar surface and responsible for the production of surfactant, and type I pneumocytes, which are derived from type II cells and cover the rest of the alveolar surface.

The surface layer of the trachea and proximal bronchi is formed by ciliated columnar epithelial cells, interspersed with goblet cells. Submucosal glands are numerous at this level and they consist of "sacs" of invaginated epithelium. Each gland comprises four distinct regions; 1) a short funnel-shaped ciliated duct, a continuation of the surface epithelium; 2) a non-ciliated collecting duct; 3) mucous tubules, lined with mucous secretory cells, opening in the collecting duct; 4) serous tubules, lined with serous secretory cells, opening into the mucous duct (199;242). Serous cells are responsible for

the secretion of water (the sol phase of the airway surface liquid – ASL) and antimicrobial peptides, while mucous cells secrete mucins (the gel phase of ASL).

Airway mucus is derived from submucosal glands and from goblet cells lining the epithelium. Mucus hypersecretion is an important feature in many chronic airway diseases, including chronic obstructive pulmonary disease (COPD), CF, and asthma (13;53). Earlier studies have shown that mucus hypersecretion is associated with airway obstruction, which is related to increased mortality (12;125). From these findings, one may conclude that excessive airway hypersecretion causes mucous plugging that contributes to airway diseases and eventually leads to deterioration and death. Since transepithelial ion secretion/absorption represents a major regulatory pathway of mucus secretion and composition, the purpose of this project was to investigate the mechanisms that regulate this process.

The airway epithelium separates two compartments: the serosal side (blood) and the mucosal side (airways lumen). To be a functional barrier, epithelium has a polarized structure, with membranes of different characteristics – the apical membrane faces the mucosal side and the basolateral membrane faces the blood vessels and adjacent cells. The resistance of airway epithelium measured in different species is around 300 Ω cm^2, which classifies this epithelium as "tight", being a poor conductor of water and electrolytes and capable of maintaining transepithelial osmotic gradients (199).

1.2.1 Airway surface liquid (ASL)

The apical surfaces of the airways are coated by a thin layer of fluid, called ASL. The composition, depth, pH and physical properties of ASL depend on many factors,

such as rate of evaporative water loss, secretion from airway submucosal glands and absorption that occurs in the airway surface epithelium. Derangements in composition and amount of ASL leads to improper cilia beating and impaired mucociliary clearance.

Although many studies have targeted the composition and the depth of ASL, there is still controversy about this information, since the results vary greatly from group to group. The most abundant and important ions in physiological fluids are Na^+, K^+, Ca^{2+}, Cl^- and HCO_3^-; therefore, most efforts were put towards measuring the concentration of these ions in ASL. Two of the main problems encountered by researchers in their quest to accurately determine the concentration of these ions in ASL were the small amount of fluids involved and the high chance of injuring the epithelium during experiments. High K^+ concentrations found in some studies indicate that surface epithelium cells may have been damaged (141;299). Another main criticism was the identification of a greater amount of ASL then expected (5 $\mu l/cm^2$ of airway surface for approximately 50 μm thin ASL) (137). Many techniques have been used to determine the ASL composition, such as X-ray microanalysis (144), microelectrode methods (37), in vivo microdialysis (97;98), radiotracers (323) or fluorescent probes (128;270); according to these studies, ASL is either isotonic (97;98;128) or hypotonic (323).

Airway epithelial cell cultures grown on semipermeable plastic supports at air-liquid interface have represented a possible way to overcome the technical difficulties encountered by in vivo measurements of ASL composition. Since 1976, when Madin-Darby canine kidney (MDCK) epithelial cells were used to measure vectorial ion and water transport in a polarized epithelial monolayer (191), many types of epithelial cells have been used to assess ion secretion or absorption (311). However, the ASL depth

measured in airway epithelial cell cultures is smaller (25 μm) than in intact mammalian airways, and there is greater variability between values obtained due to different conditions used for cell growth. Moreover, these cells are not subjected to the physiological processes that continuously regulate ASL in the airways (127).

By altering intracellular ion concentrations and membrane permeability, airway epithelial cells are able to maintain their negative membrane potential. The membrane potential depends primarily on Na^+ and K^+ ions, which have their gradients maintained by the Na^+/K^+ pump. Since it was established that in non-excitable cells the membranes are more permeable for K^+ than Na^+, in airway epithelial cells K^+ plays an essential role in determining the membrane potential. The change in Cl^- and HCO_3^- intracellular concentrations do not play a significant role in determining the membrane potential, since the active transport of anions is less important than of cations; the opposite works in this case, so Cl^- and HCO_3^- concentrations are determined by the membrane potential. The relationship between membrane potential and ion concentration is determined by the Goldman-Hodgkin-Katz equation:

$$E = \frac{RT}{F} \ln \frac{P_K[K]_O + P_{Na}[Na]_O + P_{Cl}[Cl]_I + P_{HCO_3}[HCO_3]_I}{P_K[K]_I + P_{Na}[Na]_I + P_{Cl}[Cl]_O + P_{HCO_3}[HCO_3]_O}$$

where E is the membrane potential, R is the gas constant, T is the temperature, F is the Faraday constant, P_x is the permeability of the membrane to the ion and [x] is the concentration of ion x either inside ($_I$) or outside the cell ($_O$).

Maintaining proper hydration of the ASL and especially of the periciliary or sol layer is essential for the proper function of mucociliary clearance. This can be achieved

by manipulation of ion concentrations by airway epithelial cells. The mucus from the gel layer absorbs any excess fluid in the periciliary layer; therefore the only variation that might occur would be a reduced periciliary layer (48). Since the periciliary layer is approximately equal to the height of the cilia (6 μm) and is the optimal environment for the cilia, cilia beating is impaired due to contact with the viscous gel layer if the periciliary layer is diminished (3;273).

The ionic composition of the ASL influences not only the mechanical capacity of airway epithelium to defend against pathogens, but also its capacity to produce small anti-microbial molecules. An increase in NaCl concentration in ASL inhibits activity of defensins against bacteria (54;93;268). An acidic pH of the ASL has a similar effect on defensins function and decreases the microbicidal activity of airway epithelial cells (203).

1.2.2 Ion channels present in airway epithelium

1.2.2 A Chloride channels

Chloride is the most abundant anion present in the human body under physiological conditions and its movement is regulated by several families of ion channels, such as voltage-gated chloride channels (CLC), CFTR (cystic fibrosis transmembrane conductance regulator), CLCA (calcium dependent chloride channels) and ligand-gated chloride channels. In airway epithelium at least three types of chloride channels are present: CFTR (145;256), CLCA (156) and CLC-2 (58;165).

The gene was cloned in 1989 and CFTR was identified (243;246). CFTR, a member of the ATP-binding cassette (ABC) transporter family (140), consists of two transmembrane domains (TMD1 and TMD2), each domain is formed of six α- helical

transmembrane segments. Both N- and C-termini are located inside the cell, and the C-terminus of each TMD contains a nucleotide binding domain (NBD), which is the ABC region from which the family derives its name. The regulatory domain of CFTR is called the R domain, which joins the C- terminus of NBD1 to N-terminus of NBD2 and contains consensus motifs for binding Protein Kinase A (PKA) (45). CFTR is a highly regulated protein, containing consensus motifs for PKC and PKA in sites other than the R domain. CFTR can bind through PDZ-domain (existing on the C-terminus) to other proteins, such as ezrin (194;257), suggesting that protein-protein interaction plays an important role in anchoring CFTR to the apical membrane and in regulation of its function.

CFTR-mediated Cl^- current is voltage independent, has a linear current-voltage (I-V) relationship in symmetrical chloride concentrations and a conductance of 6-10 pS (17;285). The anion permeability sequence is $Br^- \geq Cl^- > I^- > F^-$ or $I^- > Br^- > Cl^- > F^-$, depending on the configuration of the patch clamp, whole cell (5) or single-channel measurements (284).

CFTR is expressed in the apical membrane of lung epithelium at every level, from nasal epithelium to alveoli. However, the highest amount of CFTR is in serous cells from submucosal glands, and it is likely that this correlates with the role played by this channel in CF (72;318). In smaller bronchi, where CF pathology is believed to begin, there are no submucosal glands; instead, there are Clara cells, which are secretory cells that express the majority of the CFTR at this level (47;72;147).

Although many efforts have been made to decipher the function and structure of CFTR, there is controversy around some findings. CFTR can transport ATP (227;238) which could activates purinergic receptors and subsequently the outwardly rectifying

chloride channel (ORCC), a channel presumed to be regulated by CFTR (255). However, several studies contradict the observation that CFTR conducts or releases ATP (102;103), and it was suggested that the release of ATP was due to mechanical artifacts during patch clamp recordings, or because of cellular damage during ATP bioluminescence assays. It was also demonstrated that in epithelial cells of sweat glands or in Calu-3 cells (a model of serous cells from airway submucosal glands), CFTR does not play a role in ATP conductance (237).

In 1998 Linsdell and Hanrahan proposed a new hypothesis that the anionic antioxidant tripeptide glutathione is transported by CFTR (164). This effect may account for the high concentrations of glutathione that have been measured in the ASL (40). The loss of this pathway for glutathione transport in CF patients may contribute to the oxidative stress observed in the lung in CF.

Another matter of debate is the oligomerization state of the CFTR channel. After expression of CFTR in *Xenopus* oocytes and studies involving electron microscopy of the plasma membrane (164), it was concluded that CFTR forms a dimer to be functional as a channel (179). However, the absence of dominant negative mutations along with the finding that in co-immunoprecipitation experiments no evidence of oligomerization was found (73) strongly indicates that CFTR is a monomeric functional unit.

The CLC family of ion channels comprises nine members; the first one (CLC-0) was cloned from the electric organ of the marine electric ray *Torpedo marmorata* in 1990 by Jentsch (131). The structure of these channels has been resolved by crystallography and the three-dimensional structure of bacterial CLC protein revealed a dimer, in which each monomer has one pore (double-barreled channels) (69). Some of the CLC channels

are integral plasma membrane proteins (CLC-1, CLC-2 and CLC-K), while the rest reside mostly in intracellular membranes. Under certain circumstances, CLC-4, CLC-5 and CLC-7 can reach the plasma membrane. Most of these channels are gated in a voltage-dependent fashion (130).

The only plasma membrane CLCs present in lung epithelial cells are CLC-2 and CLC-K (195;198). CLC-2 is ubiquitously expressed and its disruption in mice leads to retinal and testes degeneration (23). The halide selectivity CLC-2 is $Cl^- > Br^- > I^-$ (288) and has a relative low conductance of 2-3 pS (173;306). This channel can be activated by hyperpolarization (288), low pH in the extracellular medium (132) and cell swelling (101). CLC-2 channels display an inward rectification, are blocked by 5 nitro-2-(3-phenylpropilamino) benzoic acid (NPPB), Zn^{2+} or diphenylamine-2-carboxylic acid (DPC) and are not influenced by phosphorylation (83). CLC-2 channels are reported to be poorly blocked or resistant to DIDS (83;288).

CLC-K channels are comprised of two isoforms (CLC-Ka and CLC-Kb) with a high degree of homology (90%). Their main site of expression is the kidney (130). To be functional as chloride channels, CLC-K need an auxiliary subunit, called barttin, which forms heteromers with CLC-K (18). Mutations in CLC-Kb cause Bartter syndrome type III, characterized by dehydration with late onset, whereas mutations of barttin, which co-localizes with CLC-K channels on the basolateral plasma membrane of the renal tubules and in the potassium secretory epithelia of the inner ear, cause Bartter syndrome typeIV, characterized by sensorineural deafness and renal failure (18;74;258). CLC-K channels, along with barttin, have been identified recently in human airway epithelial cells, but their precise physiological role in this tissue has to be established (195).

The halide selectivity of CLC-K1 channels is $Br^- > Cl^- > I^-$ (294;303), while for the CLC-K2 is $Br^- > I^- > Cl^-$ (2). Both isoforms display outward rectification (130), and the conductance is about 7 pS when they are expressed without barttin and of 20 pS when barttin is co-expressed (252). Currents conducted through these channels are decreased by lowering the extracellular pH and are increased by enhancement of extra cellular Ca^{2+} (303). CLC-Ka is sensitive to blockade by 2-(p-chlorophenoxy)-3-phenylpropionic acid and DIDS, but CLC-Kb is approximately six times less sensitive to both compounds (160;221).

Ca^{2+}- dependent chloride channels have been described functionally in epithelium from airways (6;50;302), intestine (319) or pancreas (325). Typically the currents exhibited by these channels are outwardly rectifying and can be blocked by DIDS or niflumic acid. The activation of these channels may or may not require phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII). The halide permeabilities reported vary greatly, as well as conductances (from 1 to 70 pS), indicating a diversity of molecular candidates for these channels (130;206).

Regarding the molecular candidates, Ran and Benos proposed that CLCA family members are putative calcium dependent chloride channels (232;233). Members of this family are expressed in human lung (202). However, there are several studies which imply that the current generated by CLCA would be not due to their properties as chloride channels, but rather to their ability to activate endogenous chloride channels (100;245). Another criticism was the high amounts of Ca^{2+} (2 mM) used to stimulate these channels in patch clamp experiments (88;100).

CLCA may be an alternative for defective CFTR and studies in CF mice models confirmed an upregulation of the Ca^{2+}-dependent chloride channels in the lung and protection against the pulmonary manifestations of CF (99;286). These channels are activated by intracellular calcium, or other factors, such as bradykinin or ATP, that lead to elevation of calcium inside the cell (99). However, apparently they cannot take over the function of CFTR and prevent the disease (25;192). One possible explanation for why CLCA are not sufficient to replace CFTR would be their lack of ability to inhibit ENaC as CFTR does (183), although even this effect has been questioned lately (200).

Recently, a new member of the chloride channel family, bestrophin, has been proposed to function as a calcium dependent chloride channel (14;223;281). Since the function of this channel in airway epithelial cells is one of the main topics of this project, detailed comments about its structure and regulation will be given throughout this thesis with a focus in Chapter 4.

1.2.2 B Sodium channels

ENaC was cloned initially using mRNA from rat distal colon, expressed in *Xenopus* oocytes (38). Later on, ENaC was also cloned from human kidney (186). This channel is a multimeric protein which is comprised of three homologous subunits, named alpha, beta and gamma (39). The alpha subunit alone can generate current, whereas beta or gamma subunits cannot conduct current by themselves (4). ENaC has low conductance (5 pS), a high Na^+ over K^+ selectivity and it is specifically blocked by amiloride at low concentration (micromolar range) (212). The activity of ENaC is regulated by different molecules, such as the ubiquitin ligase Nedd4 via ubiquitination and decrease in the

number of channels at the cell surface (1;94;274), aldosterone via increase in the expression of the channel (8;300) and by serum- and glucocorticoid-inducible kinase (sgk) (44;204;216).

ENaC is expressed in the apical membrane of most epithelia with high transepithelial resistance (4). In the respiratory tract it is expressed from nasal passages to the alveoli (75). In the alveolar epithelium one role of ENaC is well established, many studies showing that it is responsible for the physiological removal of the fetal lung fluid after birth (11;118;209).

An attractive hypothesis for the pathology encountered in CF is the interaction between ENaC and CFTR. Studies from Boucher's laboratory demonstrated that the overexpression of the ENaC beta subunit in mice causes enhanced Na^+ absorption and subsequently the CF phenotype (175). It was suggested that CFTR has an inhibitory effect on ENaC and the presence of functional CFTR is necessary for the down-regulation of ENaC (279). However, these studies could not be confirmed in other epithelia where ENaC and CFTR are co-expressed, such as sweat gland epithelium or *Xenopus* oocytes (200;234;236). In an attempt to resolve conflicting evidence, Kunzelmann et al. proposed that CFTR and ENaC do not interact directly, but rather through some molecules which are specifically expressed in certain epithelia (148).

1.2.2 C Potassium channels

Potassium channels have been studied extensively, especially for their role in stabilizing the membrane potential. Among the first who investigated potassium channels were Hodgkin, Huxley and Katz who studied sodium and potassium conductance

underlying the action potential in the giant axon of the squid (116;117). A major discovery in this field was with the use of X-ray crystallography that revealed the structure and the selectivity filter of the pore that goes through the center of potassium channels (65).

Potassium channels can be grouped in three classes depending on the number of transmembrane domains (TM): 1) channels characterized by six TM, such as Ca^{2+} activated K^+ channels or voltage-gated K^+ channels; 2) channels which consist of four TM, such as two-pore-domain channels; and 3) channels comprising of two TM, such as ATP-dependent K^+ channels or G-protein-coupled K^+ channels (129). Despite their molecular diversity, potassium channels contain in all their subunits, regardless of the number, a conserved sequence of amino acids (threonine, valine, glycine, tyrosine and glycine) called the K^+ channel signature (112). Due to their rather high single channel conductance and availability of specific blockers, potassium channels are relatively easy to study by patch clamp. However, in clinical practice there are only a few openers and inhibitors available; the only drugs widely used in the clinic are the sulfonylurea receptors blockers, which inhibit ATP-dependent K^+ channels and stimulate the release of insulin in patients with diabetes.

In airway epithelium there are several members of the K^+ channels family present, such as KCNQ1 (also known as KvLQT1, voltage-gated K^+ channel, mutated in long QT syndrome type 1), KCNN4 (an intermediate-conductance Ca^{2+}-activated K^+ channel) and the two-pore-domain potassium channels (56;57;176;283). While KCNQ1 and KCNN4 are present on the basolateral membrane (57), the two-pore-domain potassium channels are likely to be located on the apical membrane (59). To be functional, KCNQ1 requires

several accessory subunits, such as minK (encoded by KCNE1 gene) (280), MiRP1 (encoded by KCNE2 gene) (291) and MiRP2 (encoded by KCNE3) (253). In Calu-3 cells, only KCNE2 and KCNE3 have been identified (57), which suggests that KCNQ1 has distinct biophysical properties in these cells. KCNQ1 co-assembles with MiRP3 to form a cAMP-activated basolateral K^+ channel in both airway and intestinal epithelium (26;176;253). Basolateral K^+ channels have a well established role in enhancing anion secretion, especially upon activation by either Ca^{2+} or cAMP (56;63;307).

1.3 The role of basolateral Cl channels in airway epithelial cells

In Calu-3 cells, Cl^- enters the cells across the basolateral side via a $Na^+/K^+/Cl^-$ cotransporter (NKCC), a secondary active transport process driven by electrochemical gradients established by the basolateral Na^+/K^+ ATPase. Cl^- secretion is achieved by movement of Cl^- through apical chloride channels, the major one being CFTR. K^+ enters also through basolateral NKCC and exits via basolateral K^+ channels. HCO_3^- entry is through basolateral Na^+/HCO_3^- cotransporter (NBC) and it exits also via CFTR in the apical membrane.

The basolateral Cl^- channels are thought to have a role in additional entry of Cl^- and to contribute to an even larger secretion of Cl^- (119). It was also suggested that some of these channels are responsible for recycling part of the Cl^- that enters via the NKCC and regulate in this way the amount of Cl^- and HCO_3^- that is secreted (282). Future studies will be necessary to clarify the role of basolateral Cl^- channels and to determine their importance under physiological or pathological conditions.

Although the presence of basolateral Cl^- conductance is well established in airway epithelium, the molecular identity of these Cl^- channels has been unclear. Based on conductance, selectivity, and regulatory mechanisms I hypothesized that bestrophin could be a molecular candidate for the basolateral Cl^- channels in airway epithelium. Bestrophin was shown to be inhibited by chloride channel blockers such as DIDS, SITS and niflumic acid (223;281), to be activated by ATP (14;46) or calcium (228;293) and to localize to the basolateral membrane of RPE (177). Bestrophin protein mutations were shown to be responsible for the pathology of vitelliform macular dystrophy (VMD) or Best's disease (10;36;111;220).

1.4 Chloride channelopathies of epithelial cells

1.4.1 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive transmitted disease, caused by mutations in the CFTR gene located on chromosome 7. As a result of these mutations, CF patients present with a variety of manifestations, such as persistent pulmonary infection most frequently by *Pseudomonas aeruginosa* or *Staphylococcus aureus*, nasal polyposis, pancreatitis, malabsorption, meconium ileus, increased concentration of salt in the sweat gland secretions, failure to thrive and infertility. There are approximately 850 CFTR mutations known to cause the disease, but the most common one is the ΔF508, in which phenylalanine is deleted.

The mutations in CFTR gene can be divided in five classes, based on their effects on protein synthesis, trafficking and function (161):

Class 1 – mutations that produce no protein due to a stop mutation or fatal errors in the CFTR mRNA synthesis; as a result there is no cell surface Cl^- transport

Class 2 – mutations in which CFTR is transcribed and translated, but due to folding defects it does not traffick to the membrane (ΔF508 is not properly folded, and although is functional, it is destroyed prematurely in the endoplasmic reticulum)

Class 3 – mutations in which CFTR is produced and reaches the plasma membrane, but there is a defective channel regulation (it does not respond to cAMP) and as a result there is reduced or absent Cl^- transport

Class 4 – mutations in which CFTR reaches the plasma membrane and is responsive to cAMP, but it has reduced conductance

Class 5 – mutations in which there is a reduced synthesis of the protein, resulting in non-sufficient number of channels in the plasma membrane.

Currently there are several hypotheses that link CFTR malfunction to airway disease in CF (24;299;313). As expected, at the hub of these hypotheses is ASL composition or pH.

In what was called the "high-salt hypothesis", airway epithelium regulates the volume of liquid in the mucus by the hypotonic transport (312;323). According to this hypothesis, in normal individuals the salt concentration in ASL is low, while in CF there is decreased absorption of salt from ASL, and the high content of salt inhibits the activity of natural anti-microbial peptides such as defensins (93;268). This hypothesis is largely based on Ussing chambers experiments with airway epithelial cells under open-circuit conditions, by that meaning that there is no flow of ions and transepithelial potential differences are measured. In this model it is presumed that bacterial colonization occurs

before the changes in viscosity of mucus in CF. The hypotonic model is based largely on a high resistance epithelium, such as sweat duct epithelia or monolayers of airway epithelial cells that reached a resistance of ~ 800 Ω cm^2; however, this model may not be valid for human airway epithelium, which has a resistance of ~ 300 Ω cm^2

The "low-volume hypothesis" states that there is an isotonic absorption of sodium and anion secretion (183). This model is based on Ussing chambers experiments in which I_{sc} is measured with the transepithelial potential clamped at zero. In CF, due to hyperactivity of ENaC and consequent Na hyperabsorption, mucus becomes dehydrated and viscous, impairing its clearance and promoting bacterial proliferation (175). Although this model assumes that water follows salt absorption via paracellular pathways, it was shown that silencing AQ5 (aquaporin) diminishes the secretion of submucosal glands, implying the existence of a transcellular pathway for water (22). Another drawback of this model is that it assumes a transepithelial potential equal to zero, which is not what occurs under physiological conditions.

The "low pH hypothesis" postulates that CF ASL has a lack of HCO_3^-, which leads to an acidic environment and perturbation of the mucociliary clearance mechanism (51;230;235). It is still not well understood why in certain epithelia, including airway epithelium, there is preferential secretion of HCO_3^- over Cl^-, despite the fact that Cl^- is more abundant than HCO_3^- and CFTR is more permeable to Cl^- than to HCO_3^- (122;123;225).

The "low oxygenation hypothesis" postulates that the thickened mucus in ASL along with the increased consumption of oxygen by airway epithelial cells lead to a reduced amount of oxygen in the AS and to increased bacteria growth and impaired

clearance in CF (316). As a mechanism it was proposed that the hyperactive ENaC contributes to the increased oxygen consumption, by increasing the absorption of Na^+ and subsequently dehydrating the mucus component of ASL.

The "defective gland function hypothesis" postulates that the cause of pulmonary disease in CF is reduced fluid secretion by the airway submucosal glands and potentially altered secretion of mucous glycoproteins (126;292). In support of this hypothesis are the observations that CFTR is mainly expressed in serous cells lining the ducts in submucosal glands (318) and that CF mouse model fails to mimic the pulmonary disease likely due to paucity of airway submucosal glands (104). According to this hypothesis there is not only a reduced volume of ASL, but also increased viscosity and alterations in pH, ionic content and protein concentration (299).

Currently there is no curative treatment for a patient with CF. The lung disease is considered the main target, since this is the cause of death in 90% of the patients. Many efforts to find the cure have been put towards rescuing the ΔF508 CFTR and finding a non-toxic opener for the mutant channel. Another possible therapeutic avenue is gene therapy, but the results so far have been disappointing. Interestingly, after lung transplantation, these patients tend to develop more frequently infections with *Pseudomonas aeruginosa* than non-CF lung transplant recipients (208). It has been suspected that there might be a CFTR-mediated immunological defect (222) or a defect that originates in stem cells of airway epithelial cells (304), but this has to be rigorously evaluated..

4.2 Vitelliform macular dystrophy (Best's disease, VMD)

Macular dystrophy is an autosomal dominant disease, caused by mutations in the VMD_2 gene located on chromosome 11 (11q13). The VMD_2 gene was cloned in 1998 from families with Best's disease (178;220), an early-onset form of macular degeneration, in which the primary defect is thought to be in the RPE. Best's disease is characterized by typical yolk-like lesions in the retina, due to accumulation of lipofuscin within and beneath RPE, and abnormal electroculogram recordings. Lipofuscin is comprised of oxidatively modified proteins and lipids and has a fluorescent component – N-retinylidene-n-retinylethanolamine (A2E), which allows it to be detected in fundoscopic examinations. In RPE, due to its localization mainly in the lysosomal system, lipofuscin is believed to represent an incomplete degradation product of outer segments of photoreceptors (271).

Best's disease is part of a larger family of diseases characterized by macular degeneration. Age-macular degeneration (AMD), the most common cause of blindness in western countries, is also characterized by the accumulation of lipofuscin. Due to the resemblance between AMD and Best's disease, the study of bestrophin-1 (the product of VMD_2 gene) polarized the interest of the research community. Human bestrophins are members of the evolutionary highly conserved RFP-TM family, first described in *Caenorhabditis elegans* as hypothetical transmembrane proteins (276). Each of these proteins has a unique C-terminus, and the degree of homology in the family is around 65%. Nathans et al. demonstrated that expression of human, *Drosophila* and *Caenorhabditis elegans* bestrophin homologues in HEK 293 cells elicits Ca^{2+}-dependent

Cl^--currents and that mutations associated with Best's disease reduce or abolish the membrane current (281).

Several observations suggest that bestrophins can form oligomeric chloride channels: 1) Best's disease is associated with a diminished slow light peak wave of the electrooculogram (19;172;287), which is thought to be due to a Cl^- conductance across the basolateral membrane of retinal membrane epithelium (86;87); 2) bestrophin was reported to localize on the basolateral membrane of retinal pigment epithelium (177); 3) hydropathic plot of bestrophin suggests a protein with several transmembrane domains (228;276;281) 4) all the mutations in bestrophin associated with Best's disease are dominant and most of them cluster in or close to the predicted transmembrane domains (10;178;220;309).

Based on several studies demonstrating that VMD_2 gene is mutated in Best's disease and the product of this gene, bestrophin, acts as a chloride channel, VMD was included in the growing family of chloride channelopathies. However, there is no treatment for this disease. Hopefully, future experiments will identify specific bestrophin chloride channel openers that could treat young patients affected by progressive loss of vision.

2. Nitric oxide

The first who opened the road for the discovery of nitric oxide (NO) was Robert Furchgott. In 1980 he made the remarkable observation that endothelial cells have to be present to obtain arterial relaxation after application of acetylcholine (82). Without

knowing the structure, he named this unstable molecule released by endothelial cells "endothelial-derived relaxing factor" (EDRF). In 1987 two groups demonstrated that EDRF is NO (120;213). For the discovery of NO Murad, Ignarro and Furchgott were awarded the Nobel Prize in 1998. Unfortunately, Salvador Moncada was omitted, although his contributions were considered fundamental and of equal importance by much of the research community (150).

2.1 NO signaling

Cyclic GMP (cGMP) was discovered shortly after cAMP, over 30 years ago (9). It was suggested to have similar functions as cAMP, mainly due to structural and biochemical similarities. Almost 15 years later, NO was shown to be a potent activator of guanylate cyclase (GC), the enzyme responsible for generation of cGMP (7;134). The time gap between the discovery of cGMP and NO was probably one of the major factors in the delay in elucidation of the physiological functions of cGMP.

The production of NO in mammalian cells is due to the activity of nitric oxide synthases (NOS), which catalyzes the oxidation of L-arginine to L-citrulline. Although the precise mechanism by which NOS catalyzes this reaction is not known, several cofactors have been demonstrated to regulate NOS activity: flavins (FAD and FMN), NADPH, tetrahydrobiopterin (BH_4) and calmodulin (149;184;185;278). Based on the site of expression and regulation of activity, NOS can be classified as: 1) neuronal constitutively expressed NOS (NOS-I or nNOS) found mainly in neural tissue (184;251); 2) endothelial constitutively expressed NOS (NOS-III or eNOS) (224); and 3) inducible NOS (NOS-II or iNOS), isolated from macrophages or hepatocytes after induction with

certain cytokines (114;315). The constitutively expressed NOS enzymes are dependent on Ca^{2+} and calmodulin, while iNOS is Ca^{2+}-independent.

NO exerts its effects not only at the site of synthesis, but also in nearby tissues, since it diffuses easily through plasma membranes. Therefore, there is no specific receptor for NO. One important molecular target for NO is GC, through which NO puts forth many of its physiological actions. GC is active in both soluble and particulate fractions of cell homogenates and it mediates the enzymatic conversion of GTP to cGMP (49;109).

The particulate form of GC is found in the plasma membrane and in different intracellular organelles, including nucleus, mitochondria or endoplasmic reticulum (138;139). There are several isoforms described and each of them contains a single transmembrane domain, with the catalytic domain located intracellularly. The catalytic domain is highly conserved among isoforms, while the extracellular domains have little similarities, except for GC-A and GC-B, which have atrial natriuretic peptide (ANP) binding abilities (81). So far, particulate GC has been shown to have a role in increasing Cl^- secretion in intestine (81) and to be involved in restoration of cGMP in the retina after activation of photoreceptors (142).

The soluble guanylate cyclase (sGC) is found in the cytoplasmic fraction of all mammalian cells and binds NO with high affinity through its heme moiety (121). It consists of two subunits (α and β), which form a heterodimer (110). Interestingly, the carboxyl termini of both subunits bear high resemblance to the intracellular domain of particulate guanylate cyclase and have catalytic activity (290).

A common signal transduction pathway in many biological systems is the formation of cGMP from GTP. cGMP is involved in modulating the activity of other proteins or

enzymes, such as cGMP-dependent protein kinases (PKG), cGMP-regulated phosphodiesterases (PDE) or cGMP-gated ion channels (15;163;201). There are two major isoforms of cGMP–dependent protein kinases: PKGI, found in cytoplasm, and PKGII, which is membrane-bound (60;92;162). Both isoforms are homodimers (the subunits have a molecular weight of 76 kDa) and they are almost identical in the carboxyl termini (where the cGMP binding site and the catalytic domains are), but display little homology in their N-termini (60;92). PKGI is found mainly in vascular and non vascular smooth muscle, platelets or cerebellar Purkinje cells, while PKGII has been described in the brush border of intestinal microvilli. The two isoforms have a greater affinity for cGMP than cAMP (100-fold higher). Although their structure is very similar, the physiological functions assigned are different. PKGI is involved in modulation of Ca^{2+} homeostasis and consequently smooth muscle relaxation and platelet anti-aggregatory effects (55;71). PKGII inhibits active Na^+ and Cl^- absorption, but stimulates electrogenic Cl^- secretion (207). Specifically, PKGII is found in close proximity to CFTR and stimulates it more potently than PKGI (80).

The physiological responses to cGMP are regulated also by a family of PDE, which are capable of hydrolyzing the cyclic nucleotides to biologically inactive 5'-nucleotides. Thus, these enzymes play an essential role in the amplitude of response once the cGMP has been produced. There are at least six isoforms of PDE (16), but only PDE type V and VI have been shown to be cGMP specific (79;159)

Cyclic GMP-gated ion channels are an example of protein kinase independent actions of cGMP. These channels are particularly important in photo-transduction and in olfactory signal transduction. In rod photoreceptors, there is a signaling cascade that

causes hydrolysis of cGMP by PDE type VI and as a result, cGMP-gated channels close. Following closing of these channels, a transient hyperpolarization and a neural signal occur. In olfactory epithelium a cGMP-dependent channel resembling the photoreceptor channel has been identified (64), with an important role in odor sensing (31).

Another way through which NO exerts its physiological actions is via S- nitrosylation of proteins, which is the modification of protein Cys thiols by NO (113). S-nitrosylation resembles phosphorylation in many ways and specific amino acids are targeted by this modification (151). Some of the most important enzymes involved in the production of S-nitrosothiol (SNO) are NOS and ceruloplasmin (95;124). SNO breakdown is also regulated, as in the example of S-nitrosoglutathione (GSNO), which is reduced to GSH and ammonia in the presence of GSNO reductase (229).

2.2 The role of nitric oxide in human airways

NO is normally present in exhaled air from humans, although its exact source has not been identified (68). All three isoforms involved in NO production have been identified in the respiratory tract and it is presumed that they play a role in NO production (305). NO has a short half-life in vivo (1-5 s), mainly because of its reactivity with different physiological substrates (170), but its exact role in the airways is poorly understood (240;241).

eNOS transcript and protein levels increase during fetal lung development, suggesting a role in lung growth and maturation (135;214). One of the effects of NO that is ubiquitous is relaxation of the smooth muscle (133). NO is involved in airway smooth muscle relaxation via activation of sGC, production of cGMP and activation of PKG, as

in other tissues. cGMP-dependent mechanisms also involve a decrease of Ca^{2+} release, due to inhibition of inositol triphosphate and ryanodine receptors from the sarcoplasmic reticulum by cGMP (133). NO activates BK channels via sGC and PKG and subsequent membrane hyperpolarization inhibits Ca^{2+} entry through voltage-dependent Ca^{2+} channels, contributing to airway smooth muscle relaxation (321). In addition to cGMP-dependent pathways, another mechanism proposed is the direct S-nitrosylation by GSNO of unspecified proteins that regulate airway smooth muscle contraction (219). GSNO is present in nano to micromolar concentration in normal airways and its level varies greatly in pathophysiological states (90).

In asthmatic patients exhaled NO is increased (182), in parallel with an overexpression of iNOS (249). NO concentration in the airways can be used as a marker for asthma exacerbations and the reduced levels of exhaled NO are an indicator of glucocorticoid effectiveness in asthma treatment (215;263). Interestingly, low levels of GSNO have been detected in asthmatic patients and it was suggested that this contributes to refractory bronchospasm (70;91).

Modulation of ion channels in the airway epithelium by NO affects the composition and volume of ASL, the mucociliary clearance and the capacity to fight infection (68;240). NO activates apical CFTR and basolateral potassium channels, increasing anion secretion in airway epithelial cells (43;67). Although both cGMP dependent and independent pathways are involved, it seems that cGMP plays a more important role in the regulation of ion channels in airway epithelium (68). As a result, alterations in NO production in the airways may contribute to the pathogenesis of lung disorders characterized by a hypersecretion of ASL.

In CF, the exhaled levels of NO are reduced, likely due to a deficiency of iNOS expression in the airways (96;136). Although CF is characterized not only by a defect in ion channel function but also by airway inflammation, there is no strong correlation between the exhaled levels of NO and the severity of symptoms in CF patients (289). However, when CF patients received GSNO by aerosol, an increase in oxygen saturation and in exhaled NO levels was observed (269). Some beneficial effects of GSNO in the airways might be due to its ability to relax airway smooth muscle, enhance ciliary motility, inhibit amiloride-sensitive sodium transport and prevent pathogen replication.

3. Plasma membrane composition

3.1 The role of cholesterol in the plasma membrane

Singer and Nicholson described the plasma membrane "fluid mosaic model", in which it is presumed that proteins are floating between two layers of lipids (262). Several studies have addressed the composition and the contribution of each constituent to the plasma membrane. The general acceptance of this model implies that plasma membrane proteins need to be surrounded by lipids, but the specific role of the lipids and the nature of interactions between membrane proteins and lipids have yet to be elucidated.

Cholesterol is a major constituent of the plasma membrane in mammalian cells, and it has been associated with many regulatory functions of the membrane proteins. Cholesterol is particularly increased in the plasma membrane, although it is present also in intracellular membranes of mitochondria and endoplasmic reticulum (152). Glycerophospholipids are present in all cellular membranes, while cholesterol and

sphingolipids are present mainly in the plasma membrane (107;298). Sphingolipids have a moiety of ceramide and either a phosphocholine head group (sphingomyelin) or a carbohydrate structure (glycosphingolipids). The exact percentage of different lipids in the plasma membrane is not known and varies among different cell types (196). However, attempts have been made to measure the concentrations of these lipids and according to these studies cholesterol constitutes 30-40 mol % (153), sphingomyelin represents 10-20 mol% and glycosphingolipids are usually negligible in most cells, but in intestinal epithelia they make almost 30 mol % of the plasma lipids (155;261).

There are several hypotheses trying to explain why the plasma membrane is enriched in chlolesterol. An appealing theory is the one that involves the high affinity of the sphingolipids for cholesterol (231). Like cholesterol, sphingomyelin is enriched in the cell membranes and it maintains the high cholesterol concentration (153;266). Degradation of sphingomyelin decreases the cell membrane cholesterol content by mobilizing cholesterol for esterification and reducing its biosynthesis (105;265;266). Another interesting hypothesis involves caveolin, a protein found in caveolae, with cholesterol-binding properties and involved in cholesterol shuttle to the cell membrane (197;267). According to Fielding et al. cholesterol first reaches the caveolae, and then it is redistributed to the rest of the plasma membrane (78).

Since the plasma membrane has two leaflets (intracellular and extracellular), the preferential localization of the lipids has been studied. Cholesterol has a high rate of flipping between the two leaflets (275) and seems to be found equally in both compartments of the plasma membrane. Most of the sphingolipids and phosphatidylcholine are found in the outer leaflet, while part of the glycerophospholipids

(phosphatidylethanolamine or phosphatidylserine) is mainly concentrated in the inner leaflet (32;231).

Liver is an important source of cholesterol, since it is the main site of expression of hydroxymethylglutaryl-CoA reductase (HMG CoA reductase), a rate limiting enzyme in cholesterol synthesis. Cholesterol is carried mainly by two lipoproteins, low density lipoprotein (LDL) and high density lipoproteins (HDL). Cholesterol is carried from the liver to other tissues by LDL and the binding of LDL to LDL receptors is mediated by apolipoproteins. After uptake by the cells, LDL is delivered to the lysosomes, where cholesteryl esters are hydrolyzed to release free cholesterol. The reverse process of transporting the excess or the unused cholesterol from the cells to the liver is mediated by HDL, with the involvement of apolipoproteins as well.

An interesting parallel has been made between CF and diseases characterized by a defective transport or synthesis of cholesterol. Niemann-Pick disease type C (NPC) is an autosomal recessive disease, characterized by accumulation of unesterified cholesterol in the endosomal and lysosomal system, mostly due to a defect in NPC1 gene (95% are due to mutations of NPC1, the rest is due to mutations of NPC2) (20;41;218). Interestingly, in cell culture models of CF and NPC iNOS expression is reduced and statins, inhibitors of cholesterol synthesis, are able to correct this defect by inducing expression of iNOS (146;310). Another interesting pathological condition, Tangier disease is characterized by an impaired offloading of cholesterol from the cells and reduced HDL, due to mutations in ABCA1, a member of the ATP binding cassette (34;239). Interestingly, mutations or reduced expression of ABCA1 cause pulmonary lesions and increased levels of

cholesterol precursors in the lung (66;187;297). Further studies need to be done to shed light on the role played by cholesterol regulation in the pathology of CF.

Cholesterol level in the plasma membrane affects the physical properties of the lipid bilayers. Addition of cholesterol to the cell membrane makes the lipid acyl chains tightly packed and the bilayer thicker and more rigid (322). Cholesterol can also order the bilayer in one dimension and reduce the permeability (211). Accordingly, the high-cholesterol bilayer was named "liquid-ordered" versus "liquid-disordered" state without cholesterol (196). The liquid-ordered domains are also known as lipid rafts.

3.2 Lipids rafts/caveolae

Simons and Ikonen defined lipid rafts as plasma microdomains enriched in cholesterol and sphingolipids (259). Although sphingolipids are mainly present on the outer leaflet of the cell membrane, the raft hypothesis refers to both leaflets. However, presently there is no clear explanation about how lipid rafts in the extracellular leaflet can regulate the signal transduction molecules found in the cytoplasmic leaflet of the plasma membrane (196). The highly-ordered structure of lipid rafts makes them insoluble in detergents (Triton X-100), while the surrounding plasma membrane is soluble (33).

The size of a raft is approximately 50 nm, which corresponds to about 3500 molecules of sphingomyelin (226). Due to the small size, a raft can accomodate probably no more than 10 to 30 proteins (260). Some proteins are considered markers of lipid rafts, simply because they have been found constantly associated with lipid rafts; among these ones there are 5-nucleotidase (277), placental alkaline phosphatase (33),

hemagglutinin (264) and GM1 ganglioside (108). All these proteins are anchored in lipid rafts by glycosylphosphatidylinositol (GPI) (108).

Lipid rafts are seen as platforms for different signaling molecules, which couple with receptors, enzymes or ion channels. Tyrosine kinase receptors, such as those for endothelial growth factor (190) and insulin (296), are localized in lipid rafts. G protein-coupled receptors, such as adenosine A_1 (154), bradykinin (61;248), β–adrenergic (247) and M2 muscarinic (76) receptors reside in lipid rafts. Among signaling molecules found in lipid rafts, adenylate cyclase (254), Src-family kinases (168), Gα subunits of heterotrimeric G proteins (272) and eNOS (210) play an important role.

An impressive number of ion channels are associated with lipid rafts, including BK (29), Kv1.3 (21), Kv1.4 (314), Kv 1.5 (181), Kv2.1 (180), Kir 2.1 (244), Kir3.1 (62), transient receptor potential channels (30;171), ENaC (115) and cyclic nucleotide-gated channel CNAG2 (28). Although CFTR is not found in lipid rafts under normal conditions (27), in the presence of *Pseudomonas aeruginosa* it becomes raft-associated (143). Lipid rafts are characterized by continuous association and dissociation, a property that matches with the plasma membrane physiological functions of adapting to different stimuli.

Caveolae, a sub-set of lipid rafts, were identified for the first time in the 1950s, by using electron microscopy (320). Caveolae are flask-shaped plasma membrane invaginations, with a diameter of 60-80 nm, enriched in cholesterol and with similar detergent-resistance properties as lipid rafts (77). Apart from these features, a defining characteristic of caveolae is the presence of caveolin, a 21 kDa protein that forms an intra-membrane hairpin loop, with both N-and C-termini intracellularly (52). Caveolin, which is now considered a marker for caveolae, plays an important role in intracellular

cholesterol transport, in membrane traffic and in signal transduction (169). The ability of caveolin to form oligomers (167), along with the discovery of a scaffolding domain between amino acids 82 and 101 of caveolin sequence (158) aligned with the observation that caveolin clusters with several signaling molecules. eNOS, which is one of the proteins abundant in caveolae, has a caveolin-binding motif; it is believed that caveolin inactivates eNOS by antagonizing calmodulin binding and preventing Ca^{2+}-dependent activation of eNOS (35;89).

With respect to the polarized structure of the epithelium, lipid rafts are believed to play a role in apical or basolateral sorting of proteins. Although GPI-anchored proteins have been found to be localized to the apical membrane of Madin-Darby canine kidney (MDCK) epithelial cells (166) and caveolae to the basolateral side of epithelial cells (250;301), a consensus has not been reached among researchers about the exact localization of lipid rafts to either side.

4. Thesis objectives

Airway epithelial ion transport determines the composition and amount of ASL, which is a major determinant of the mucocilliary clearance apparatus. The purpose of this thesis is to investigate different mechanisms that regulate airway epithelial ion transport, with a focus on the role of basolateral chloride channels. The main goals are:

1. to identify the basolateral chloride channels at the molecular level
2. to characterize the signal transduction pathway through which these channels are activated by NO

I hope that the findings of my studies will contribute to a better understanding of the ion transport mechanisms in airway epithelium under physiological and pathological conditions.

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CHAPTER 2

The role of bestrophin in airway epithelial ion transport

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1. **Introduction**

The presence of Cl^- channels in the basolateral membrane of airway epithelial cells has been established in several studies (10;30-32), but their physiological role is not well characterized. In a search for the molecular identity of the basolateral Cl^- channels, we have considered the members of the well established (CLC, CFTR, GABA/glycine receptors), and less well-characterized (CLIC or CLCA) Cl^- channel families as possible candidates (for review see (11)). Among these, ClC-2 channels have been localized to the basolateral membrane in different cell types (4;12;33), but these channels are unlikely to play a major role in human airway epithelia because basolateral Cl^- channels are regulated via cAMP-dependent pathways (28;30), while ClC-2 channels are not (11).

Recently, a new family of putative Cl^- channels, bestrophins, has been identified in retinal pigment epithelium (RPE) (27). Bestrophin is a protein encoded by the vitelliform macular dystrophy type 2 (VMD2) gene (16;19), which is mutated in Best macular dystrophy (9). It localizes to the basolateral membrane of RPE cells (14), and constitutes a new family of Cl^- channels unrelated in primary sequence to any previously characterized channel proteins (21;22;29).

In this study we have investigated bestrophin expression in Calu-3 cells, an airway serous cell model (25), and used a small interfering RNA (siRNA) approach to transiently and specifically reduce its expression. Our findings indicate that bestrophin is present in Calu-3 cells and that ablation of its expression results in diminished apical-to-basolateral $^{36}Cl^-$ flux.

2. Material and Methods

2.1 Cell culture

Calu-3 cells were grown as previously described (28). For transepithelial measurements cells were seeded onto Costar Snapwell inserts (0.45-μm pore size), and grown submerged in culture medium for the first six days. Subsequently, air interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10-16 days after the establishment of an air interface.

2.2 RT-PCR

Forward and reverse primers for bestrophin (VMD2, GenBankTM accession number NM_004183) were nucleotides 329-348 and 516-535, respectively. As a positive control, plasmid containing VMD2 gene (a generous gift from Dr. I. McDonald, Ophthalmology, University of Alberta) was used. DNA amplification was obtained after 38 cycles by annealing for 1 min at 58°C, followed by an elongation step at 72°C for 1 minute. The size of the amplified product was 207 bp and was sequenced by the University of Alberta DNA Sequencing Core Facility to confirm its identity.

2.3 Immunocytochemistry

Western blotting was performed as described elsewhere (26). Primary rabbit polyclonal antibody against bestrophin (Bst-101AP) was purchased from FabGennix Inc., Shreveport, LA. Where appropriate, pre-absorption controls were carried out,

using the blocking peptide supplied with the antibody. Controls were also carried out in which the primary antibody was replaced with PBS; these were negative (not shown). A minimum of three runs was carried out and a representative example is shown. Optical band densities were determined using Sigma Gel software (Jandel Scientific).

2.4 Transepithelial measurements

Ussing chamber studies were performed as previously described (7). Cells grown on inserts were mounted into CHM5 Ussing chambers (WPI, Sarasota, Fl), and bathed on apical and basolateral sides with 10 ml of Krebs Henseleit Solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4 (9). Solutions were warmed to 37°C and circulated with a gas lift using 95% O_2-5% CO_2. During all experiments, the I_{sc} was allowed to stabilize for 20 min prior to treatments. In all experiments amiloride (10 μM, apical) was present in the apical compartment to inhibit ENaC-mediated Na^+ absorption. Stock solutions for 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 5 mM), S-nitroso-glutathione (GSNO, 100 mM) and amiloride (10 mM) were prepared in H_2O, whereas forskolin (10 mM) in ethanol. All chemicals were from Sigma (St. Louis, MO).

2.5 RNA interference

The selection of the coding sequence for siRNA was determined using Qiagen siRNA Designer (Qiagen, Mississauga, Ontario, Canada) and was analyzed by BLAST research to ensure that it did not have significant sequence homology with other genes. The 21-oligonucleotides purchased as ready-annealed purified duplexes from Qiagen

(sense5'CACAAGCAGUUGGAGAAACdTdT3', antisense 3'dTdTGUGUUCGUCAA CCUCUUUG-5') targeted bestrophin mRNA sequence in position 588-608. Transfection was carried out using Qiagen RNAi Starter Kit. Control siRNA was fluorescein-labeled, 21 bp of random sequences provided by Qiagen, and were also used to optimize transfection efficiency. In preliminary studies we found that the 1:9 (μg:μl) ratio of RNAiFect Transfection Reagent to siRNA, produced the best results, with over 80% of cells being transfected. The experiments were performed with cells grown to confluency at the air-liquid interface. 14.5 μl of 20 μM siRNA and 33.75 μl of transfection reagent were added to a medium in a total volume of 250 μl, and left for 15 min at room temperature to allow formation of transfection complexes. Subsequently the mixture was diluted with a complete cell culture medium to a total volume of 1 ml, added to the basolateral side, and the cells were incubated for 72 hours.

2.6 Radioisotopic Flux

Calu-3 inserts were short-circuited for 20 min prior to the addition of the radioisotope. At time zero (T_0), background samples were taken, followed by the addition of 3 μCi of $^{36}Cl^-$ (specific activity of 0.126 mCi/ml, Amersham Pharmacia Biotech, UK) to the basolateral compartment, and another 20 min allowed for the establishment of equilibrium. At this time (T_{20}) 0.5 ml samples were taken from the apical side and replaced with fresh KHS; this was repeated at 10 min. intervals thereafter. Three samples were taken (T_{20} - T_{40}) before the addition of forskolin (10 μM); this was followed by two more samples taken (T_{50} and T_{60}) before the addition of basolateral DIDS (50 μM), and a further two samples taken (T_{70} and T_{80}) after DIDS

treatment. Two samples were also taken from the basolateral side before treatment with forskolin, to calculate the specific activity. Samples were counted in a liquid scintillation counter, and the basolateral-to-apical flux (J^{Cl}_{BA}) was calculated according to standard equations (24). $^{36}Cl^-$ fluxes in the apical-to-basolateral (J^{Cl}_{AB}) direction were measured in exactly the same fashion, except that the radioisotope was added to the apical bathing solution. Net $^{36}Cl^-$ flux (J^{Cl}_{net}) was calculated as $J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$.

2.7 Statistical analysis

Results are presented as the means ± SE; n refers to the number of experiments. The paired Student's t-test was used to compare the means of two groups. Differences among the means of multiple groups were determined by one-way analysis of variance with the Tukey-Kramer post-test using Instat 3.05 (Graphpad software, San Diego, CA).

3. Results

Figure 2.1 shows that Calu-3 cells express bestrophin mRNA under normal cell culture conditions. To estimate the possible sensitivity of Calu-3 cells to bestrophin knockdown by siRNA, initial experiments were performed at 24 h post-transfection. No significant differences were revealed between control cells and cells transfected with bestrophin siRNA (data not shown). However, 72 h incubation with bestrophin-directed siRNA resulted in significant and specific suppression of bestrophin protein, as judged by Western blotting (Fig. 2.2). Measurement of band optical density indicated that the siRNA treatment reduced the bestrophin band intensity to 34.6 ± 5.8 % (n=3) of its control value.

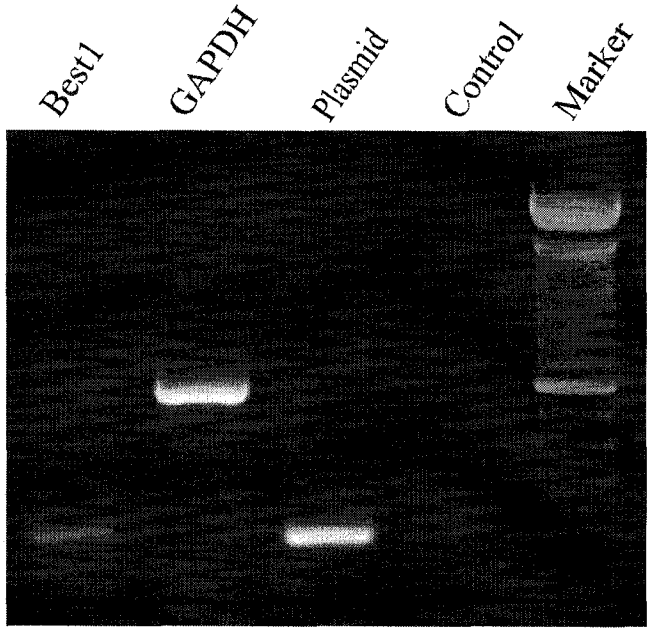

Figure 2.1 Expression of bestrophin in Calu-3 cells. *Calu-3 mRNA was reverse-transcribed into cDNA and PCR-amplified using bestrophin-specific primers (lane 1*

from left). GAPDH amplification of the same sample is shown as a control for RNA quality. Plasmid containing VMD2 gene was used as a positive control. PCR amplification was also conducted on RNA preparations (without reverse transcription) to control for possible genomic contamination (lane 4). The expected sizes of the PCR products are 207 bp (bestrophin), and 595 bp (GAPDH). Marker - DNA standard (a 100-bp ladder).

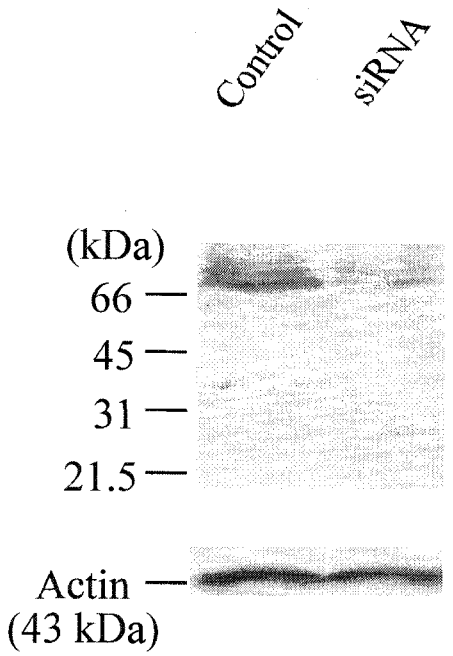

Figure 2.2. Specific knockdown of bestrophin expression in Calu-3 cells by siRNA. *Cells, cultured on 6-well plates, were subjected to transfection with either bestrophin siRNA, or control siRNA. At 72 h post-transfection cells were homogenized and representative samples were assayed by Western blotting. The blot shown is representative of three experiments, and actin served as a loading control. The bestrophin antibody labels a 68-kDa protein in Calu-3 cells. Additional bands seen may be glycosylated forms or other isoforms of bestrophin.*

The next set of experiments studied I_{sc} in Calu-3 monolayers transfected with either bestrophin siRNA or with control siRNA (Fig. 2.3). The average transepithelial resistance and baseline I_{sc} in bestrophin siRNA treated cell inserts were 356.5 ± 40 Ωcm^2 and 30.9 ± 2.7 $\mu A\ cm^{-2}$ (n=6) respectively, and were not different from control values (287.0 ± 24.5 Ωcm^2 and 41.6 ± 7.3 $\mu A\ cm^{-2}$ (n=6), respectively). Similarly, the effects of the nitric oxide donor, GSNO (100 μM), on the peak I_{sc}, were not significantly different in control and siRNA treated cells (41.1 ± 10.3$\mu A\ cm^{-2}$, n=3, and 46.6 ± 6.6 $\mu A\ cm^{-2}$ n=6, respectively). However, subsequent application of DIDS (50 μM, basolateral) increased I_{sc} by 25.6 ± 3.5 $\mu A\ cm^{-2}$ (n=3) under control conditions, but only by 7.4 ± 1.1 $\mu A\ cm^{-2}$ (n=6, $P<0.01$) after siRNA treatment. These results suggest that bestrophin siRNA affects the function of basolateral DIDS-sensitive Cl^- channels.

A direct way to show that bestrophin siRNA treatment affects chloride secretion is to measure chloride movement using $^{36}Cl^-$. Unidirectional $^{36}Cl^-$ fluxes were measured in both the basolateral to apical (J_{BA}) and in the apical to basolateral (J_{AB}) directions. Figure 2.4 shows the net $^{36}Cl^-$ flux rate (J^{Cl}_{net}) during each sample interval in control and siRNA-treated cells. This information is supplemented with unilateral $^{36}Cl^-$ flux data in Table 2.1. Consistent with the findings from earlier studies (6; 28), the data confirm that under basal conditions there is no net movement of $^{36}Cl^-$ in Calu-3 cells. Interestingly, cells treated with bestrophin siRNA showed higher baseline and forskolin stimulated net $^{36}Cl^-$ fluxes than control cells (Table 1, T_{50} and T_{60}). Subsequent application of DIDS (50 μM, basolateral) decreased J_{AB} from 2.92 ± 0.30 $\mu Eq\ cm^{-2}\ h^{-1}$

to 1.30 ± 0.46 μEq cm^{-2} h^{-1} (P<0.05) under control conditions, but had no significant effect on $^{36}Cl^-$ flux in siRNA treated cells (Table 1, T_{70} and T_{80}).

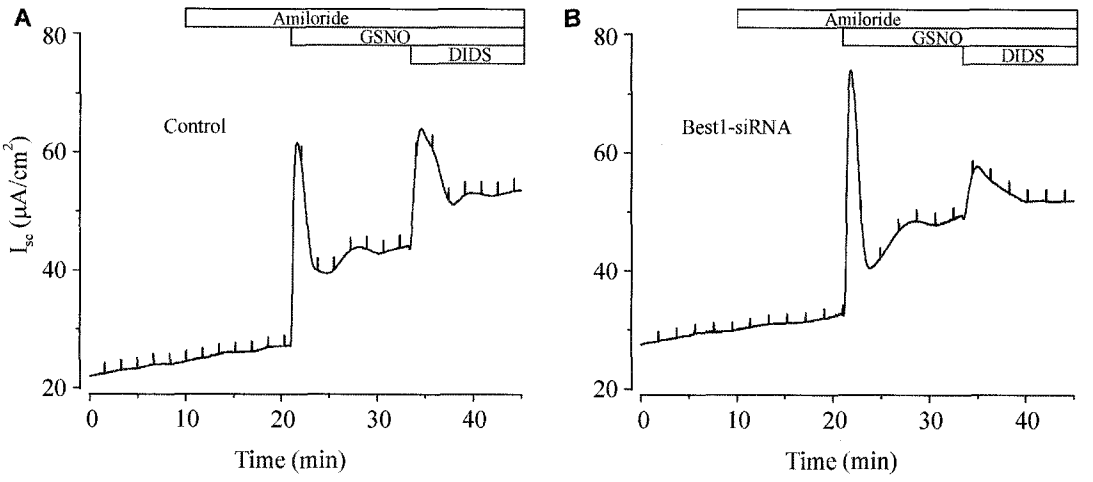

Figure 2.3 Bestrophin siRNA reduces DIDS-sensitive basolateral Cl^- conductance in Calu-3 cells. *Representative recordings (siRNA-treated n=6; control n=3) show I_{sc} responses to GSNO (100 μM, bilateral) and DIDS (50 μM, basolateral). The effect of DIDS on GSNO-activated I_{sc} is reduced by more than 70 % (from* 25.6 ± 3.5 *μA cm^{-2} to*7.4 ± 1.1 *μA cm^{-2}, n=6) in bestrophin vs. control siRNA treated cells.*

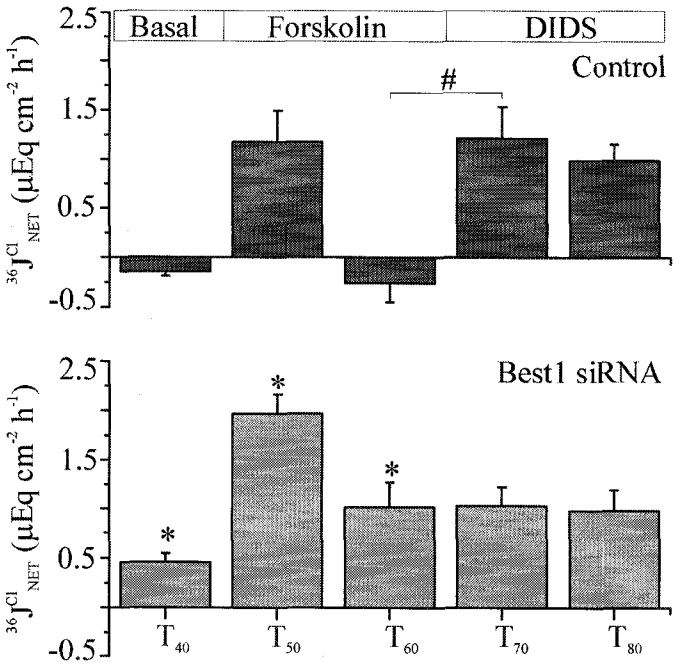

Figure 2.4 Net $^{36}Cl^-$ fluxes in control and bestrophin siRNA-treated cells. *Measurements were performed under basal conditions (T_{40}), in the presence of 10 μM forskolin (T_{50} and T_{60}), and 50 μM DIDS (T_{70}, T_{80}). Net fluxes were obtained from six experiments. $^{*}P<0.05$, fluxes in control and siRNA-treated cells. $^{\#}P<0.01$, effect of DIDS under control conditions.*

Sample	Treatment	Control			Bestrophin siRNA-treated		
		J_{BA}	J_{AB}	J_{NET}	J_{BA}	J_{AB}	J_{NET}
T_{40}	Baseline	0.98 ± 0.21	1.11 ± 0.11	-0.13 ± 0.18	1.41 ± 0.23	0.95 ± 0.08	0.46 ± 0.09 *
T_{50}	Forskolin	3.57 ± 0.60	2.40 ± 0.23	1.17 ± 0.32	4.46 ± 0.38	2.48 ± 0.23	1.97 ± 0.19 *
T_{60}	Forskolin	2.67 ± 0.23	2.92 ± 0.30	-0.26 ± 0.19	3.18 ± 0.31	2.16 ± 0.40	1.02 ± 0.16 *
T_{70}	+ DIDS	2.51 ± 0.44	1.30 ± 0.46	1.21 ± 0.32	3.28 ± 0.23	2.25 ± 0.32	1.03 ± 0.26
T_{80}	+ DIDS	2.31 ± 0.31	1.33 ± 0.14	0.98 ± 0.17	2.80 ± 0.26	1.81 ± 0.33	0.98 ± 0.21

Table 2.1 Unidirectional and net ion fluxes across short-circuited Calu-3 monolayers. *Flux values are in $\mu Eq\ cm^{-2}\ h^{-1}$. Forskolin (10 μM) was added bilaterally, DIDS (50 μM) was added basolaterally. Each unidirectional flux was obtained from n=3 experiments, therefore net fluxes from n=6 experiments.* * *$P<0.05$, significantly different from the corresponding flux in control cells.*

Discussion

Since its discovery in *C. elegans* (8), RNA interference has become an effective method for the analysis of gene function (18). We used this technique to study the bestrophin gene-function relationship in human airway epithelial cells. Our results suggest that bestrophin functions as a basolateral Cl^- channel regulated via cAMP- and cGMP-dependent phosphorylation.

Current models of bestrophin topology suggest that this protein consists of either four (1;27) or six (21;22) transmembrane spanning α-helices and a large C-terminal cytoplasmic region, containing four potential phosphorylation sites for protein kinase A, two for protein kinase C, and three for cGMP-dependent protein kinase (PKG) (2). In earlier studies we had shown that GSNO stimulates transepithelial anion secretion acting via a cGMP-dependent pathway (7). More recently, basolateral Cl^- channels were shown to be activated via cAMP-dependent phosphorylation (28). The results of this study show that silencing the bestrophin gene with siRNA decreases GSNO-activated, DIDS-sensitive I_{sc}, and forskolin-activated $^{36}Cl^-$ flux. This suggests that bestrophin functions as a basolateral Cl^- channel regulated via cAMP- or cGMP-dependent phosphorylation. There is also the possibility of cross talk between cAMP- and cGMP-dependent pathways because cAMP inhibits cGMP-specific phosphodiesterase 5, and both cAMP and cGMP can activate PKG (23). Bestrophin is known to interact with protein phosphatase 2A (15), and phosphorylation/dephosphorylation of its C-terminal could act as the on/off switch regulating channel activity.

Western blot experiments suggest that siRNA treatment reduces bestrophin expression in Calu-3 cells by ~65%. However, measurements of $^{36}Cl^-$ flux indicate that bestrophin siRNA reduces basal J_{BA} by only ~14%. This suggests that bestrophin may not be the only Cl^- channel present in the basolateral membrane. There is significant evidence that ubiquitously expressed ClC-2 Cl^- channels are present in the basolateral membrane of epithelial cells (4;5;12;33), and these channels could also contribute to the basolateral Cl^- conductance.

Although bestrophins were initially shown to function as the Cl^- channels in RPE cells (22;29), there is now significant evidence to suggest that these channels could also play an important physiological role in other tissues. Boese et al. (3) have shown that Cl^- conductances in collecting duct cells have some important similarities to bestrophin-induced conductances (3). Others have described cGMP-activated Cl^- channels in smooth muscle cells that share some characteristics with the bestrophin channel family (17;20). Interestingly, recent studies with a member of the CLCA channel family, pCLCA1, have found that although the CLCA protein localized to the basal RPE, its primary function was to regulate the activity of other proteins rather than to mediate Cl^- conductance (13). Although the results of our study strongly suggest that bestrophin functions as a basolateral Cl^- channel, we cannot exclude the possibility that this protein may function as a regulatory component of an ion channel-protein complex. However, recent experiments showing that mutations of bestrophin residues in a putative channel pore alter the relative permeability and conductance for different anions (21) provide strong evidence that bestrophins function as ion channels, rather than regulators of channel activity.

In conclusion, the results of our studies show, for the first time, that bestrophin is present in airway epithelial cells and functions as a basolateral Cl^- channel. We also show that bestrophin channel activity in Calu-3 cells is regulated via cAMP- and cGMP-dependent pathways. Basolateral Cl^- channels have previously been proposed to play a crucial role in determining the magnitude of Cl^- and HCO_3^- secretion (28), and the knowledge of their molecular identity may have important clinical implications.

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CHAPTER 3

Regulation of basolateral Cl^- channels in airway epithelial cells: the role of nitric oxide

This chapter is in press:

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Background

Under normal conditions, the airway epithelium functions as a highly selective barrier that protects the lungs from potentially harmful substances. To facilitate this task, the epithelium has developed a number of protective mechanisms that include changes in transepithelial ion transport and altered mucociliary clearance. Anion secretion provides a driving force for hydration of the airway surface liquid. This process is coordinated by a polarized network of channels, transporters, and energy-dependent pumps that are selectively expressed in the apical and/or basolateral aspects of the epithelium. In airway epithelial cells the primary basolateral entry pathways for Cl^- and HCO_3^- are the Na^+-K^+-$2Cl^-$ (NKCC1) and the Na^+-HCO_3^- cotransporters, respectively. Both anions leave the cell via the apical Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) (5).

While apical membrane Cl^- channels have been studied intensively and much is known about their role in transepithelial anion transport, the basolateral Cl^- channels have received significantly less attention. The presence of basolateral Cl^- channels in the airway epithelium was first reported over 15 years ago (29), but little is known about their role in transepithelial anion secretion (13). The fact that inhibition of these channels increases Cl^- secretion indicates that at least some of the Cl^- ions that enter the cell via NKCC1 cotransport are recycled across the basolateral membrane (27).

Presently, at least two different types of Cl^- channels, have been identified in the basolateral membrane of epithelial cells: ClC-2 and bestrophin. ClC-2 Cl^- channels are broadly expressed, are insensitive to DIDS, and are activated by hyperpolarization, cell swelling, and acidic pH (14). In addition to the basolateral

membrane localization (3), ClC-2 channels have also been shown to be present in the apical membrane (20) and at the apical junctional complexes in epithelial cells (12). Several physiological functions have been proposed for ClC-2 (14), but their role in transepithelial ion transport in the human airway remains unknown.

Bestrophin was initially identified as the protein product of the gene responsible for autosomal dominant vitelliform macular dystrophy (VMD), also known as Best disease (18;21). VMD is associated with an irregular electrooculogram, thought to reflect changes in the retinal pigment epithelium (RPE) basolateral membrane Cl^- conductance (8). Although bestrophin mRNA and protein are highly enriched in the RPE (21), this channel is also present in other epithelia, including airway epithelial cells (7). Current models of bestrophin topology suggest that this protein consists of either four (21;26) or six (22) transmembrane spanning α-helices and a large C-terminal cytoplasmic region containing three potential phosphorylation sites for protein kinase A, and for cGMP-dependent protein kinase (PKG) (2). The fact that basolateral Cl^- channels could be activated via cAMP- and/or cGMP-dependent phosphorylation (7), suggests that bestrophin could play a major role in the regulation of transepithelial anion secretion in human airways.

Activation of the nitric oxide (NO)/cGMP-dependent pathway is known to stimulate anion secretion in the human airway (6) and lung epithelial cells (15). NO is produced by three NO synthases (NOS), but current evidence indicates that the respiratory tract NO is produced mainly by inducible NOS (iNOS), with little contribution from endothelial (eNOS) and neuronal (nNOS) isoforms (16). Moreover, iNOS expression can be upregulated many fold by inflammatory agents such as

cytokines, leading to generation of large amounts of NO in the airways (1). Therefore, we investigated the effects of endogenous and cytokine-induced NO on transepithelial Cl^- secretion. Our results indicate that Calu-3 cells primarily secrete HCO_3^- when stimulated via the NO/cGMP pathway, and that Cl^- secretion can be stimulated by blocking basolateral Cl^- channels. We also show that bestrophin Cl^- channels are broadly expressed in the human lung, not only in airway epithelial cells, but also in alveolar macrophages, endothelial cells and airway smooth muscle cells.

Methods

Cells

Calu-3 cells were obtained from the American Tissue Culture Collection (Rockville, MD), and grown as described previously (Szkotak et al., 2003). Briefly, cells were maintained in T-75 flasks (Costar, Cambridge, MA), and typically required 6-8 days to reach ~85% confluence. At this time the cells were seeded at a density of 3.5×10^5 cells/cm^2 onto Costar Snapwell inserts (0.45-μm pore size, 1 cm^2 surface area) for short-circuit current (I_{sc}) measurements, and maintained in a cell culture medium containing 20% FBS. For the first 6 days, cells were grown submerged in culture medium. Subsequently, air-liquid interface culturing was used, in which the medium was added only to the basolateral side of the inserts. Inserts were used for experiments 10-16 days after the establishment of an air-liquid interface.

Transepithelial Measurements

Cells grown on inserts were bathed on their apical and basolateral sides with 10 ml of Krebs-Henseleit Solution (KHS) containing (mM): 116 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose, pH 7.4. Solutions were warmed to 37°C and continually circulated with a gas lift using either 95% O_2-5% CO_2 if the solution was HCO_3^- buffered, or air if the solution was HEPES buffered. In experiments requiring HCO_3^--free KHS the composition was (mM): 135.8 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 10 Hepes, pH 7.4. In low Cl^- solution NaCl (116 mM) was replaced by Na gluconate (112 mM), and $CaCl_2$ (2.5 mM) was replaced by Ca gluconate (5 mM) to compensate for the

Ca^{2+}-buffering capacity of gluconate. The transepithelial potential difference was clamped to zero using a DVC 1000 amplifier (WPI, Sarasota, Fl) and the resulting I_{sc} was recorded through Ag-AgCl electrodes and 3 M KCl agar bridges. I_{sc} was sampled at 10 Hz using a PowerLab 8SP series data acquisition converter and Chart software, both from ADInstruments (NSW, Australia). Brief (1 s) pulses of 0.5 mV were applied every 90 s, to calculate resistances. All values are expressed as an average ΔI_{sc}, which was calculated as the mean change in current in the first 300 s after drug addition, unless otherwise noted. During all experiments, the I_{sc} was allowed to stabilize for 20 min prior to treatments.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting

PAGE and Western blotting were carried out as described previously (24). Briefly, cells were homogenized in protease inhibitor buffer containing 15 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml pepstatin, and 1.74 mg/ml phenylmethyl-sulphonyl fluoride (PMSF). Protein concentration was determined using the Bio-Rad Bradford-based protein assay method and 50 μg of protein was added to each well of a 10% polyacrylamide gel. Proteins were transferred from the gels onto a supported nitrocellulose membrane at 100 V for 2 h. For immunoblotting, membrane blocking was carried out using 5% skimmed milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T). Primary antibodies were incubated overnight at 4 °C followed by four washes for 10 min each, after which the peroxidase conjugated secondary anti-rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) was used in a dilution of 1/10000 in TBS-T for 1 h at room temperature. Proteins were visualized by adding

ECL Western blotting detection reagent for 10 s and then exposing the blot to Hyperfilm ECL (Amersham, Baie, Quebec, Canada). Where appropriate, pre-absorption controls were carried out, using the control blocking peptide supplied with the antibody. Pre-absorption abolished staining in all cases (not shown). Controls were also carried out in which the primary antibody was replaced with normal rabbit serum; these were also negative (not shown). A minimum of three runs was carried out and a representative example is shown.

Immunoprecipitation was performed with cell extracts collected in modified RIPA buffer (Upstate, Waltham, MA). The cell lysate was pre-cleared on protein A sepharose beads (Sigma, St. Louis, MO) at 4 °C for 15 min, and then 0.5 mg of protein was incubated with bestrophin antibody for 2 h followed by incubation with protein sepharose A beads overnight. The next day the beads were washed 3 times with PBS and the immuno-complexes recovered by boiling with 30 µl 2x SDS buffer containing 5% β-mercaptoethanol. The samples were analyzed by Western Blot and probed with antibody that recognizes serine, threonine and tyrosine phosphorylated proteins.

Radioisotopic Flux

Calu-3 inserts were short-circuited for 20 min prior to the addition of the radioisotope. At time zero (T_0), background samples were taken, followed by the addition of 3 µCi of $^{36}Cl^-$ (Amersham Pharmacia Biotech, UK) to the basolateral compartment, and another 20 min allowed to establish equilibrium. At this time (T_{20}) 0.5 ml samples were taken from the apical side and replaced with fresh KHS; this was

repeated at 10 min intervals thereafter. Three samples were taken (T_{20}, T_{30} and T_{40}) before the addition of S-nitroso-glutathione (GSNO, 100 μM); this was followed by two more samples (T_{50} and T_{60}) taken before the addition of basolateral DIDS (50 μM), and a further two samples (T_{70} and T_{80}) taken after DIDS treatment. Two samples were also taken from the basolateral side before treatment with GSNO, to calculate the specific activity. Samples were counted in a liquid scintillation counter, and the basolateral-to-apical flux (J^{Cl}_{BA}) was calculated according to a standard equation (25). $^{36}Cl^-$ fluxes in the apical-to-basolateral (J^{Cl}_{AB}) direction were measured in exactly the same fashion, except that the radioisotope was added to the apical bathing solution. Net $^{36}Cl^-$ flux (J^{Cl}_{net}) was calculated as $J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$.

Immunohistochemistry

Human lung histopathological material was obtained from the University of Alberta Hospital, Edmonton, Alberta, Canada. Tissue used was a surgical specimen, representing visually normal lung. The study was approved by the Health Research Ethics Board, University of Alberta. After fixation, lung tissue was embedded in paraffin and sectioned at 4 μm. All steps were performed at room temperature in a humidified container to prevent tissue dehydration. Sections were deparaffinized, rehydrated, and endogenous peroxidase was blocked with 30% H_2O_2 - methanol (1:4) (vol/vol) for 10 min. Goat serum (Vector Lab, Burlingame, CA) was used to block non-specific binding, and a previously optimized concentration of primary antibody (51 μg/ml, Bestrophin E6-6, sc-32792, Santa Cruz Biotech., Santa Cruz, CA) in antibody diluent (Dako, Via Real Carpinteria, CA) was immediately applied to the

sections and incubated for 45 min. Unbound antibody was removed by three washes in PBS. Biotinylated anti-mouse IgG (H+L), (Vector Lab) secondary antibody (5 μg/ml) was applied to each section for 30 min, followed by 20 min incubation with horseradish peroxidase streptavidin (Vector Lab). Slides were washed in PBS, stained with 3,3'-diaminobenzidine chromogene (DAB) (Biogenex, San Ramon, CA) for 5 min and counterstained in Harris hematoxylin (Sigma). Sections were dehydrated through an alcohol series and xylene and mounted in Cytoseal XYL mounting medium (Richard-Allan Sci., Kalamazoo, MI). Antibody localization was detected by brown staining. As negative controls we used the same dilution of purified mouse IgG (Cedarlane, Hornby, ON, Canada).

Chemicals

Stock solutions of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 5 mM), N^G-nitro-L-arginine methyl ester (L-NAME, 100 mM), GSNO (100 mM), 8-Br-cGMP (50 mM), and amiloride (10 mM) were prepared in H_2O. H-89 (5 mM) was dissolved in 50% ethanol, anthracene-9-carboxylic acid (9-AC, 10 mM) in 0.1 N NaOH and 1400W (10 mM) in methanol. ODQ (1H-[1, 2, 4] oxadiazolol-[4, 3-a] quinoxalin-1-one, 10 mM), KT5823 (1 mg/ml), and nystatin (90 mg/ml) were prepared in DMSO. Ouabain (10mM) was prepared in KHS. L-NAME and ODQ were purchased from Alexis Biochemicals (San Diego, CA), DIDS from Molecular Probes Inc. (Eugene, OR), KT5823 and 1400W from Calbiochem (San Diego, CA), and all other chemicals were from Sigma. Goat polyclonal anti-bestrophin (C-14: sc-22027) and rabbit polyclonal anti-iNOS (H-174: sc-8310) antibodies were purchased

from Santa Cruz Biotech. Anti-phosphoserine/threonine/tyrosine mouse monoclonal antibody (ab15556) was purchased from Abcam Inc (Cambridge, MA, USA). Recombinant human cytokines IL-1β, TNFα and IFNγ were purchased from Chemicon International, Inc (Temecula, CA), and reconstituted in PBS according to the manufacturer's instructions.

Data Analysis

Data are presented as means ± SEM, unless otherwise indicated; n refers to the number of experiments. The paired Student's *t*-test was used to compare the means of two groups. Statistically significant differences among the means of multiple groups were determined by one-way analysis of variance (ANOVA) with the Tukey-Kramer posttest using Graphpad Instat 3.05 software (San Diego, CA). A value of $P<0.05$ was considered statistically significant.

Results

Nitric oxide activates basolateral, DIDS-sensitive Cl^- channels

In total, we evaluated 58 inserts bathed in normal KHS, and 24 inserts in either Cl^-- or HCO_3^--free KHS. The basal I_{sc} and R_T in KHS averaged 29.3 ± 4.1 μA cm^{-2} (range 2 - 61 μA cm^{-2}) and 396 ± 18 Ω cm^2 (range 241 - 826 Ω cm^2), respectively. The nitric oxide donor, GSNO (100 μM, bilateral) increased peak I_{sc} by 32.5 ± 2.5 μA cm^{-2} (range 21 - 48 μA cm^{-2}, n = 19). Similar results were obtained with another, biochemically different, NO donor, S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 100 μM, n=3), whereas a carrier of NO in GSNO (GSH, 100 μM, n=4), had no effect on I_{sc}. Consistently with previous findings (27), the absence or presence of amiloride (10 μM, apical) had no effect on I_{sc}, indicating that epithelial Na^+ channels do not contribute to the I_{sc} in Calu-3 cells.

A blocker of Cl^- transport, DIDS (50 μM), added to the apical side did not affect either baseline or GSNO-stimulated I_{sc} (n=6). Basolateral DIDS caused a small but significant increase in baseline I_{sc} (2.4 ± 0.3 μA cm^{-2}, n=6, $P<0.05$) and a much larger increase in cells pre-treated with GSNO (28.1 ± 1.6 μAcm^{-2}, n=14). Figure 3.1A shows a typical I_{sc} recording in the presence of GSNO and basolateral DIDS. Similar results were obtained with another Cl^- transport blocker, 9-AC (1 mM, n=4, Fig. 3.1B). These results suggest that the NO-activated, DIDS- and 9-AC-sensitive Cl^- channels are present in the basolateral membrane.

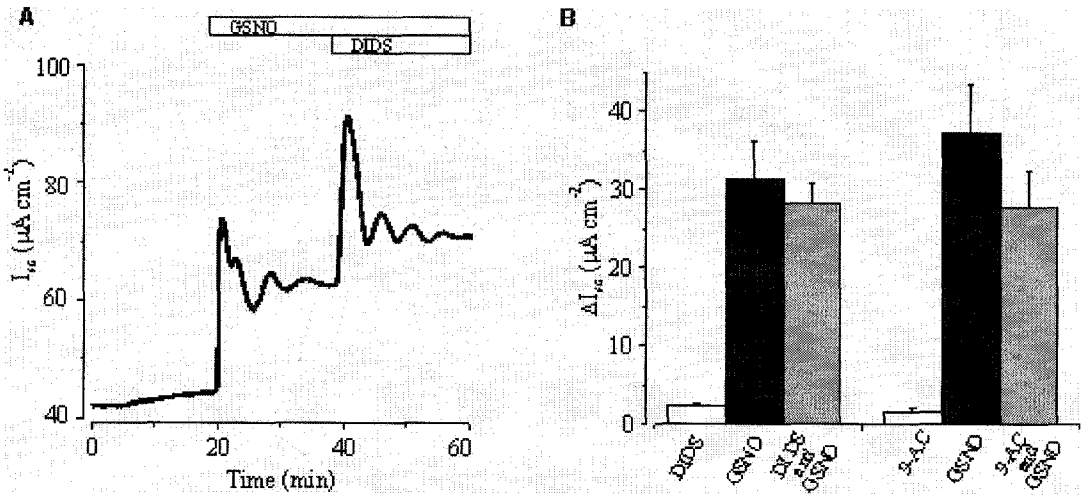

Figure 3.1. The effect of GSNO and Cl⁻ channel blockers on I_{sc}. *(A) Representative recording showing the activation of I_{sc} by GSNO (100 μM, bilateral), and basolateral DIDS (50 μM, n=14). (B) A bar chart showing averaged peak ΔI_{sc} activated by DIDS and 9-AC (1 mM, basolateral, n=4), in the absence and presence of GSNO. Addition of DIDS to the basolateral side caused a small but significant increase in baseline I_{sc} ($P<0.05$) and a much larger increase in cells pre-treated with GSNO ($P<0.0001$)*

A direct measurement of the basolateral membrane Cl^- conductance was performed using apical membrane permeabilization by nystatin (90 μg/ml). The experiments were performed in the presence of ouabain (1 mM, basolateral) to inhibit Na^+/K^+-ATPase, and in HCO_3^--free KHS to eliminate contribution of a Na^+-HCO_3^- cotransporter. Figure 3.2A shows typical current recordings in apically-permeabilized Calu-3 cells, subjected to a voltage staircase of -8 to +8 mV. The current at the beginning and end of each trace, when V was 0, is the I_{sc}. The GSNO-activated current was calculated by subtracting the baseline current from the current recorded in the presence of GSNO, and the corresponding current-voltage (I/V) relationship is shown in Fig. 3.2B. The basolateral membrane conductance was determined as the slope of the I/V curve at 0 mV. GSNO-activated basolateral conductance was equal to 1.58 ± 0.06 mS cm^{-2} (n=3). Subsequent application of basolateral DIDS (50 μM) reduced this value to 0.26 ± 0.02 mS cm^{-2}, indicating that ~84% of GSNO-activated basolateral conductance was DIDS sensitive.

ClC-2 Cl^- channels, insensitive to DIDS but blocked by Zn^{2+} ions, have been previously identified in the basolateral membrane of colonic epithelial (3) and in Calu-3 (19) cells. We investigated their contribution to the basolateral membrane Cl^- conductance by applying $ZnCl_2$ (50 μM, basolateral) in apically-permeabilized Calu-3 cells. Zn^{2+} ions decreased basolateral membrane Cl^- conductance by 0.21 ± 0.04 mS cm^{-2} (n=4). In the presence of Zn^{2+} ions GSNO increased the conductance by 1.49 ± 0.12 mS cm^{-2}, indicating that channels activated by GSNO were Zn^{2+}-insensitive.

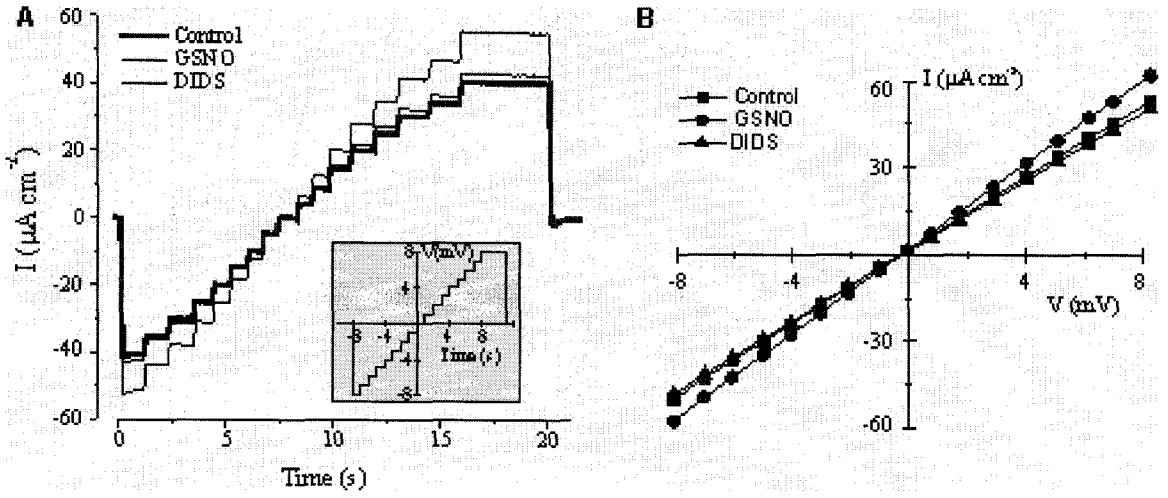

Figure 3.2. GSNO activates basolateral Cl⁻ conductance in apically-permeabilized Calu-3 cells, which is DIDS sensitive. *(A) Typical traces showing transepithelial current response to a voltage staircase of -8 to +8 mV in 1 mV steps. Cells were bathed in HCO_3^--free KHS bubbled with air, and the apical membrane was permeabilized with nystatin (90 μg/ml). The inset shows the voltage protocol used. (B) Current-voltage relationships under control conditions, in the presence of GSNO and DIDS (n=3 in each set). The standard errors varied between 3.1 and 6.8 μA cm^{-2} at ±8 mV (not shown).*

NO stimulates Cl^- but not HCO_3^- conductance in the basolateral membrane

Figure 3.3 shows typical I_{sc} responses to GSNO and DIDS in HCO_3^-- or Cl^--free solutions. In HCO_3^--free solution GSNO increased I_{sc} by 20.9 ± 4.1 μA cm^{-2} (n=4), and the subsequent application of DIDS further increased the I_{sc} by 30.9 ± 0.9μA cm^{-2} (Fig. 3.3A). This indicates that DIDS prevents Cl^- recycling across the basolateral membrane leading to enhanced Cl- secretion across the apical membrane.

In contrast, in Cl^--free solution GSNO increased the I_{sc} by 34.8 ± 2.6 μA cm^{-2} (n=4), whereas DIDS decreased the I_{sc} by 4.6 ± 1.2 μA cm^{-2} (n=4), probably due to its effects on basolateral Na^+-HCO_3^- cotransport (Fig. 3.3B). These results also indicate that basolateral Cl^- channels are impermeable to HCO_3^- anions.

In both solutions, addition of a selective inhibitor of the soluble guanylyl cyclase, ODQ (20 μM, bilateral), brought the GSNO-activated I_{sc} to the baseline levels, indicating that NO increased I_{sc} via a cGMP-dependent pathway (Fig. 3.3A,B).

Measurement of $^{36}Cl^-$ secretion reveals Cl^- recycling across the basolateral membrane

Simultaneous measurements of I_{sc} and $^{36}Cl^-$ flux were performed in Krebs-Henseleit solution. Figure 3.4 shows the averaged I_{sc} traces (n=8), the net $^{36}Cl^-$ flux (J^{Cl}_{net}) and the calculated net HCO_3^- flux ($J^{HCO_3}_{net}$) during each sample interval. The unilateral $^{36}Cl^-$ flux data are shown in Table 3.1. These data show that the majority of baseline I_{sc} is due to HCO_3^- secretion (T_{30}), with a smaller contribution from Cl^- ions. The application of GSNO (100 μM) stimulates transient Cl^- secretion (Fig. 4, T_{40}) followed by sustained HCO_3^- secretion (Fig. 4, T_{50}) and reduced J^{Cl}_{Net} (Table 1, T_{50}).

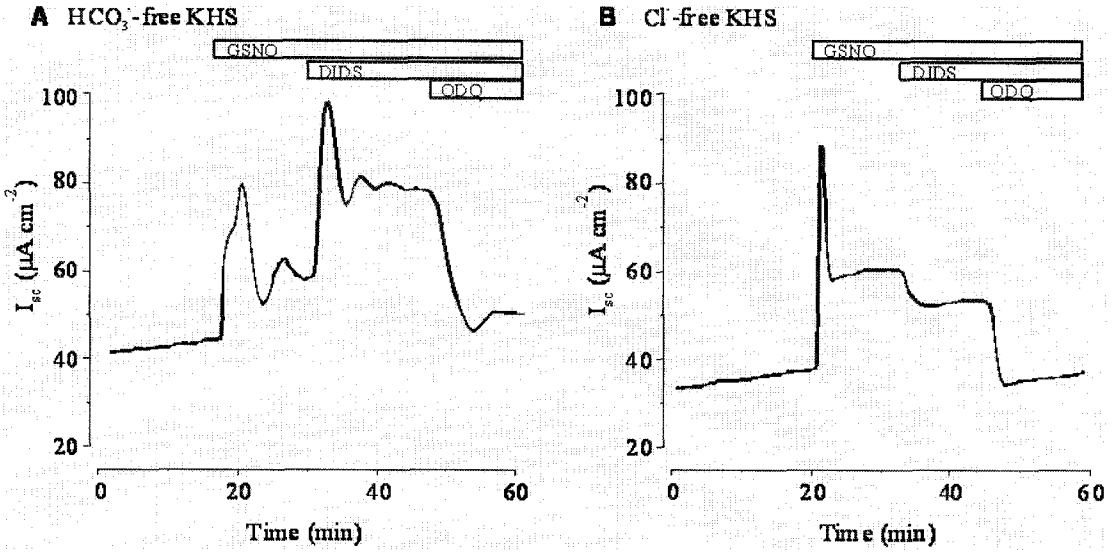

Figure 3.3 The effect of GSNO on I_{sc} in HCO_3^-- and Cl^--free solutions. *Representative recordings showing the activation of I_{sc} by GSNO (100 μM, bilateral) in (A) HCO_3^--free and (B) Cl^--free solutions, followed by DIDS (50 μM, basolateral) and ODQ (20 μM, bilateral).*

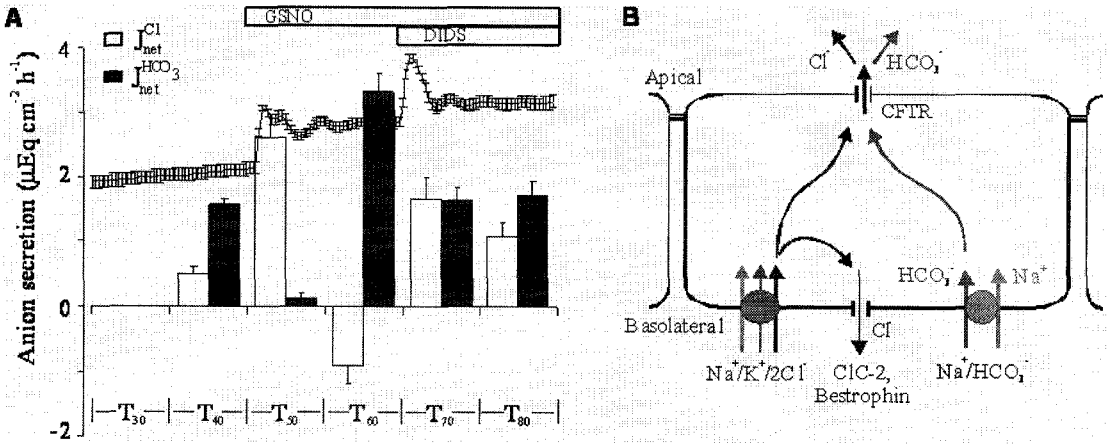

Figure 3.4 (A) Simultaneous $^{36}Cl^-$ flux and I_{sc} measurements show the relative contributions of HCO_3^- and Cl^- to Calu-3 anion secretion. *The data are expressed as a mean ± SEM (n=8). The sampling periods for radioisotopic flux measurements are shown as T_X (where X is the time in minutes). Measured $^{36}Cl^-$ net flux ($J^{Cl}_{net} = J^{Cl}_{BA} - J^{Cl}_{AB}$) and calculated HCO_3^- flux ($J^{HCO_3}_{net} = I_{sc} - J^{Cl}_{net}$) are shown for each sampling period. (B) A model of transepithelial anion secretion in airway epithelial cells.*

Sample	Treatment	J^{Cl}_{BA}	J^{Cl}_{AB}	J^{Cl}_{NET}	I_{sc}	J^{HCO3}_{NET}
T40	Baseline	1.38±0.25	0.87±0.05	0.51±0.13	2.08±0.12	1.56±0.12
T50	GSNO	4.16±0.59	1.59±0.11	2.62±0.30	2.76±0.14	0.14±0.15
T60	GSNO	1.40±0.54	2.30±0.15	-0.90±0.28	2.43±0.15	3.33±0.25
T70	+ DIDS	2.63±0.58	0.96±0.23	1.67±0.31	3.32±0.18	1.64±0.21
T80	+ DIDS	2.10±0.37	1.04±0.29	1.06±0.24	2.79±0.16	1.73±0.19

Table 3.1 *Flux values and I_{sc} are in units of $\mu Eq\ cm^{-2}\ h^{-1}$. GSNO (100 μM) was added bilaterally, DIDS (50 μM) was added basolaterally. Each unidirectional flux was obtained from n=4 experiments, while net fluxes and I_{sc} were obtained from n=8 experiments. J^{Cl}_{BA} - the basolateral-to-apical $^{36}Cl^-$ flux, J^{Cl}_{AB} - the apical-to-basolateral flux. J^{Cl}_{net} - net $^{36}Cl^-$ flux calculated as $J^{Cl}_{BA} - J^{Cl}_{AB}$.*

Subsequent measurement of $^{36}Cl^-$ flux reveals that DIDS increases J^{Cl}_{Net} by increasing J^{Cl}_{BA} and decreasing Cl^- back-flux (J^{Cl}_{AB}) across the basolateral membrane (T_{60} and T_{70}, Fig. 4 and Table 1).

A schematic diagram of anion transport in airway epithelial cells may help to better understand the effect of NO on transepithelial anion secretion (Fig. 4.4B). Under baseline conditions, CFTR-mediated HCO_3^- secretion is ~3 times greater than Cl^- secretion (Fig. 4.4A). Initially, GSNO induces a change in the (Cl^-:HCO_3^-) flux ratio, increasing the Cl^- flux ~18 times. Following this initial burst of Cl^- secretion, basolateral Cl^- channels open, prompting a switch in the CFTR-mediated anion secretion from Cl^- to HCO_3^-. Subsequent addition of basolateral DIDS inhibits Cl^- recycling across the basolateral membrane, redirecting Cl^- secretion through CFTR.

Basolateral Cl^- channels are regulated via NO/cGMP-dependent pathway

The role of phosphorylation in the regulation of basolateral Cl^- channels was investigated using a cell membrane-permeable analog of cGMP, 8Br-cGMP. Figure 3.5A shows that 8Br-cGMP (1 mM, bilateral) increased I_{sc} by 16.3 ± 5.7 μA cm^{-2} (n=3), and DIDS (50 μM, basolateral) further increased I_{sc} by 39.7 ± 11.4 μA cm^{-2}. Possible crosstalk between cAMP- and cGMP-activated pathways in basolateral Cl^- channel activation was investigated using a protein kinase A inhibitor, H-89. In the presence of H-89, GSNO increased I_{sc} by 52.9 ± 9.3 μA cm^{-2}, and DIDS further increased I_{sc} by 32.1 ±5.3 μA cm^{-2} (n=5, Fig 3.5B), indicating that basolateral Cl^- channels were activated independently of PKA activity. The role of protein kinase G

(PKG) in I_{sc} activation was investigated using KT5823, a specific PKG inhibitor. KT5823 (2.5 μM) reduced I_{sc} responses to GSNO and DIDS by 42.6% and 83.3% (n=4), respectively.

Further characterization of the basolateral Cl^- channel activation by NO was performed using apical membrane permeabilization. These experiments were performed in HCO_3^--free KHS, with an apical to basolateral Cl^- gradient (129.1: 17.1 mM), and in the presence of ouabain (1 mM), to inhibit the electrogenic Na^+/K^+-ATPase. Basolateral Na^+-K^+-$2Cl^-$ and Na^+-HCO_3^- cotransporters were inhibited by furosemide (1 mM) and HCO_3^- removal, respectively. Under these conditions, apical nystatin (90 μg/ml) evoked a strong increase in I_{sc}, revealing a basolateral Cl^- conductive pathway (Fig. 3.5C). While GSNO further increased the basolateral Cl^- current, this effect could be inhibited by ODQ indicating that basolateral Cl^- channels were activated via a NO/cGMP-dependent pathway. DIDS (50 μM) decreased the peak of GSNO-activated I_{sc} by 39.1 ± 11.9 μA cm^{-2} (n=3), and by 25.9 ± 7.5 μA cm^{-2} in the presence of ODQ (n=3, $P<0.05$ Student's *t*-test).

Bestrophin functions as a NO-activated basolateral Cl^- channel in Calu-3 cells

The presence of bestrophin Cl^- channels in the basolateral membrane of Calu-3 cells has been demonstrated earlier (7). Here, we investigated the effect of GSNO on bestrophin activity using immunoprecipitation technique. Bestrophin was precipitated from control and GSNO-treated cells using bestrophin-specific antibodies, and then precipitates were probed with phospho-specific monoclonal antibodies. Figure 3.5D shows that under control conditions bestrophin

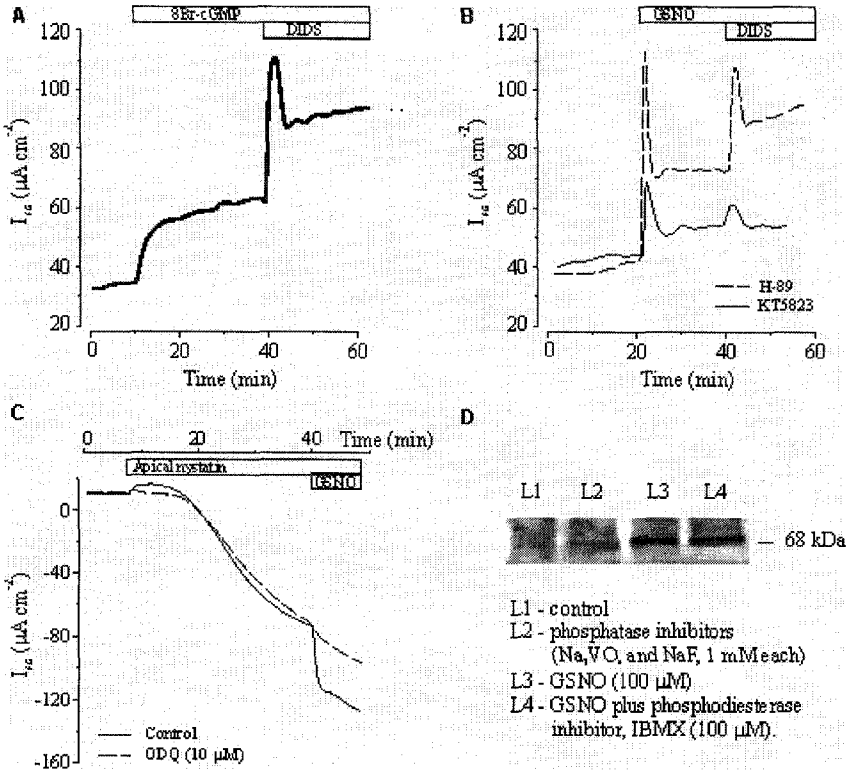

Figure 3.5. Basolateral Cl⁻ channels are activated via a cGMP-dependent pathway. *(A) In cells pre-treated with 8Br-cGMP (1 mM) there is an enhanced response to basolateral DIDS (50 μM), as compared to control cells (n=3). (B) Pre-treatment of Calu-3 cells with the PKA inhibitor, H-89 (bilateral, 10 μM), had no significant effect on GSNO (100 μM) and basolateral DIDS responses (n=5). In contrast, PKG inhibitor, KT5823 (2.5 μM), reduced I_{sc} responses to GSNO and DIDS by 42.6% and 83.3% (n=4), respectively. (C) Permeabilization of the apical membrane with nystatin reveals a basolateral Cl⁻ conductance that can be stimulated by GSNO (which is prevented by ODQ). The cells were pretreated with L-NAME (1 mM) to inhibit endogenous NO production and in the presence of ouabain (1 mM), to inhibit the electrogenic Na^+/K^+-ATPase. (D) Western blot analysis of the bestrophin immunoprecipitate with phospho-specific antibodies revealed the presence of phosphorylated bestrophin after GSNO treatment.*

was not phosphorylated (lanes L1 and L2), but became phosphorylated after GSNO treatment (lanes L3 and L4). These results suggest that bestrophin phosphorylation may play a major role in the regulation of basolateral conductance by NO.

Cytokine-induced NO activates basolateral Cl^- channels

Under baseline conditions the inducible NO synthase (iNOS) is not detected in Calu-3 cells (Fig. 3.6A). However, iNOS can be induced by incubating cells with a mixture of cytokines (TNFα, IFNγ and IL-1β, each at 5 ng/ml) for 12 hours. This treatment has no apparent effect on transepithelial resistance (277.8 ± 34.7 Ω cm^2 and 270.3 ± 11.3 Ω cm^2 in control (n=3) and cytokine-treated (n=12), respectively), but increases baseline I_{sc} from 36.8 ± 4.4 μA cm^{-2} in control cells to 48.3 ± 2.5 μA cm^{-2} in cytokine-treated cells ($P<0.01$, *t*-test). The increase in the baseline I_{sc} in cytokine-treated cells is probably related to iNOS expression, since 1400W (20 μM), a specific inhibitor of iNOS (9), had no effect on I_{sc} in control cells, but reduced I_{sc} in cytokine-treated cells to control level (Fig. 3.6C, n=3, $P<0.005$). The baseline I_{sc} in cytokine-treated cells was also reduced to control level by an inhibitor of soluble guanylyl cyclase, ODQ (Fig. 3.6D). DIDS (50 μM, basolateral) increased I_{sc} by 26.6 ± 2.4 μA cm^{-2} (n=6) in cytokine-treated cells, compared to 2.4 ± 0.3 μA cm^{-2} (n=6) under control conditions (Fig. 3.6B). The effect of DIDS on I_{sc} activation in cytokine-treated cells, in the presence of either 1400W or ODQ, was not different from DIDS effects on the baseline I_{sc} in control cells (n=3 in each set, $P>0.05$, Anova).

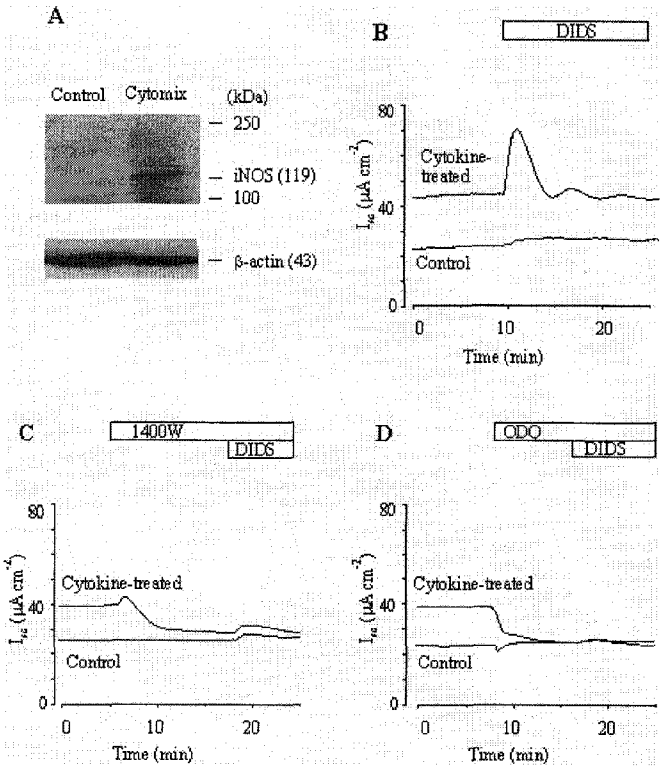

Figure 3.6 Cytokine treatment affects iNOS expression and baseline I_{sc}. *(A) Western blot showing the effect of cytokines (cytomix: TNFα, IFNγ and IL-1β, each at 5 ng/ml) on iNOS expression. iNOS was detected in cytokine-treated but not in control Calu-3 cells (n=3 in each set). (B) In cytokine-treated cells, basolateral DIDS increased I_{sc} by 26.6 ± 2.4 μA cm^{-2} (n=6), compared to 2.4 ± 0.3 μA cm^{-2} (n=6) under control conditions. (C) An inhibitor of iNOS, 1400W (20 μM, n=3), inhibited baseline I_{sc} in cytokine-treated cells but had no effect on I_{sc} in control cells. (D) An inhibitor of soluble guanylyl cyclase, ODQ (20 μM, n=3), reduced I_{sc} to control levels in cytokine-treated cells but had no effect on I_{sc} in control cells. In the presence of 1400W or ODQ, DIDS (50 μM) did not activate the I_{sc} (n=3 in each set, P>0.05, panels C and D).*

Expression of bestrophin Cl^- channels in human lung

While the expression of bestrophin in tissues such as retinal pigmented epithelium (RPE) is well documented, little is known about its presence in other tissues. In our previous study we showed that bestrophin is present in airway epithelial cells using RT-PCR and Western blot (7). Here, we used immunohistochemistry to investigate bestrophin distribution in human lung (Fig. 3.7). Specific staining for bestrophin was present in airway epithelial cells (Fig. 3.7 panel A), serous cells of submucosal glands (panel C), and alveolar macrophages (panel E). The corresponding isotype control is shown in panels B, D, and F. Positive staining for bestrophin was found also in endothelial cells and in smooth muscle cells (Fig. 3.7 panel A). Interestingly, type I pneumocytes (panel E), type II pneumocytes and also mucous cells of submucosal glands (panel C) did not show any bestrophin expression.

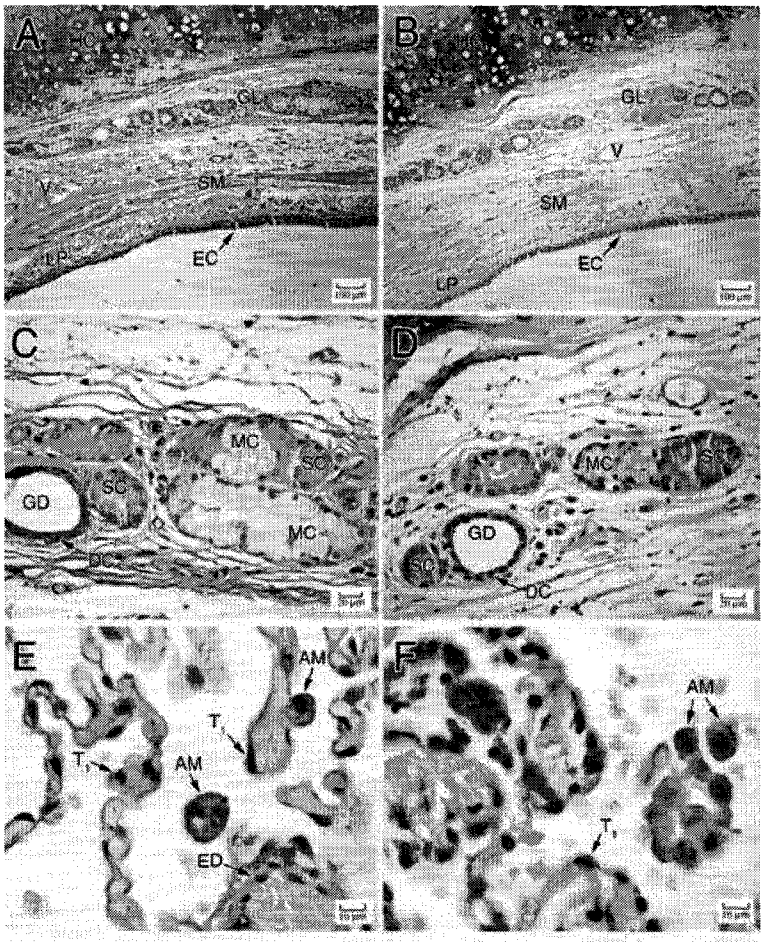

Figure 3.7 Bestrophin is expressed in human lung. *Immunohistochemistry of normal human lung sections using bestrophin antibody (panels A, C and E), and corresponding isotype controls (mouse IgG; panels B, D and F). (A-B) segmental bronchus (x100), EC - surface epithelium, HC - hyaline cartilage, LP - lamina propria, SM - smooth muscle cells, GL - submucosal glands, V - blood vessel. (C-D) submucosal glands (x400), DC - ductal cells, GD - glandular duct, MC - mucous cells, SC - serous cells. (E-F) small airways and alveoli (x1000), AM - alveolar macrophages, ED - endothelial cells, T_1 - Type I cells.*

Discussion

Nitric oxide is present in the ppb (parts per billion) range in exhaled breath in humans (17), but its physiological role in the respiratory system is uncertain (23). The results of our study show that NO is a potent activator of basolateral Cl^- channels in airway epithelial cells, especially under inflammatory conditions, when increased expression of iNOS leads to generation of large amounts of NO. This effect may reduce Cl^- secretion across the apical membrane because of increased Cl^- backflux across the basolateral membrane.

Biological actions of NO are usually classified as either cGMP-dependent or -independent (23), but many cell types appear to use both pathways (4;10). The results of this study indicate that basolateral Cl^- conductance in Calu-3 cells could be activated via the NO/cGMP-dependent pathway. This conclusion is supported by experiments showing that 1) 8Br-cGMP exerted a similar effect on I_{sc} as NO donors, 2) the effect of NO could be completely blocked by an inhibitor of soluble guanylyl cyclase, 3) a specific inhibitor of PKG, KT5823, reduced the I_{sc} response to basolateral DIDS, and 4) phospho-specific antibodies detected bestrophin in GSNO-treated, but not in control immuno-precipitates.

The sustained electrogenic Cl^- secretion in epithelia can only result from effects exerted at both the apical and basolateral poles of the epithelial cells. NO donors produce a transient increase in Cl^- secretion, suggesting involvement of additional regulatory mechanisms. Recently, GSNO has been shown to activate apically located CFTR via both cGMP-dependent and cGMP-independent mechanisms (4). Another

study suggested that GSNO may also exert inhibitory effects on CFTR channel activity by glutathionylation of cys-1344 in the second nucleotide binding domain of human CFTR (28). While the complex regulation of CFTR Cl^- channel activity by GSNO may contribute to a transient I_{sc} response, this effect alone is insufficient to explain stimulatory effects of basolateral Cl^- channel blockers on I_{sc}, following GSNO treatment.

The results of our study suggest another hypothesis of GSNO action in airway epithelial cells, based on differential activation of Cl^- channels in the apical and basolateral membranes. Simultaneous measurements of I_{sc} and $^{36}Cl^-$ flux suggest that the delay in the activation of transport processes in the apical and basolateral membranes may be responsible for the transient increase in the I_{sc} following NO treatment. GSNO may first activate CFTR in the apical membrane, which leads to an increase in the I_{sc}, and then the basolateral Cl^- channels, which causes a decrease in the I_{sc}. This hypothesis is in agreement with the results showing that GSNO stimulates transient Cl^- secretion, followed by sustained HCO_3^- secretion and reduced J^{Cl}_{Net} (Fig. 3.4A, Table 3.1). It is also supported by measurements showing that DIDS increases J^{Cl}_{Net} by increasing J^{Cl}_{BA} and decreasing Cl^- back-flux (J^{Cl}_{AB}) across the basolateral membrane (Fig. 3.4A and Table 3.1). A model of transepithelial anion transport that includes major channels and transporters identified in airway epithelial cells is shown in Fig. 3.4B.

At the present time two types of Cl^- channels have been identified in the basolateral membrane of epithelial cells, ClC-2 (3; 30) and bestrophin (7). ClC-2 channels are insensitive to cyclic nucleotide-dependent phosphorylation, are blocked

by Zn^{2+} but not by DIDS, and have conductance 2-3 pS, (14). Since GSNO increased basolateral membrane Cl- conductance in the presence of Zn^{2+} ions, ClC-2 channels are unlikely to play a role in NO-dependent regulation of the basolateral membrane Cl^- conductance. On the other hand, bestrophin is highly sensitive to DIDS (26) and contains three potential phosphorylation sites for cGMP-dependent protein kinase in the cytoplasmic C-terminal region (2). Thus, bestrophin Cl^- channels are likely to play a major role in NO-dependent regulation of transepithelial Cl^- secretion.

Immunohistochemistry studies indicate that bestrophin is present in a number of different cell types in human lung, including surface epithelial cells and serous cells of submucosal glands. Positive staining for bestrophin was found also in alveolar macrophages, endothelial cells and smooth muscle cells, but not in type I or type II pneumocytes or in mucous cells of submucosal glands. The function of bestrophin in cells such as alveolar macrophages or smooth muscle cells remains to be established.

In conclusion, our studies show that basolateral Cl^- channels are activated by NO and regulate anion secretion in Calu-3 cells. It is likely that bestrophin Cl^- channels play a major role in this process. These results may also be relevant to the function of retinal pigment epithelium, since this tissue is the main site of bestrophin expression (21) and generates NO in response to a number of cytokines (11). NO is believed to help maintain the integrity of the blood-retinal barrier, improving visual acuity (31), and this function could be accomplished, in part, through modulation of bestrophin activity.

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CHAPTER 4

Bestrophin: its function beyond vision

This chapter is a manuscript in progress for publication:

Duta, V and Duszyk, M (2006)

1. Introduction

Chloride is the most abundant physiological anion and it is distributed almost at electrochemical equilibrium in most animal cells. This distribution is facilitated by several families of chloride channels, such as ligand-gated chloride channels, cystic fibrosis membrane transport regulator (CFTR), voltage dependent chloride channels (ClC) and calcium dependent chloride channels (CaCl). Bestrophin is a newly emerged member of the chloride channels family, with a structure different than other chloride channels. Mutated bestrophin is considered to be responsible for Best's disease or macular vitelliform dystrophy, an autosomal dominant disorder characterized by a progressive loss of vision, with an early onset and a large, yellow, yolk-like (vitelliform) lesion in the central macula, due to accumulation of lipofuscin. Lipofuscin is a pigment comprising of a mixture of partially oxidized lipids and proteins and is considered to be noxious to the cells.

Bestrophin is a basolateral plasma protein encoded by the VMD2 gene (5;12) and it was proposed to function as a Ca^{2+}-dependent Cl^- channel (18;20;22). VMD2 gene was identified in 1998 and is located on chromosome 11q13 (15).

2. Structure

Bestrophin comprises of 585 amino acids, with an approximate molecular weight of 68kDa. It shares homology with the *Caenorhabditis elegans* RFP gene family, characterized by the presence of a conserved arginine (R), phenylanine (F) and proline (P) amino acid sequence motif.

Hydrophobicity profile analysis reveals that VMD2 gene product displays four strongly hydrophobic peaks at residue positions 31-50, 72-90, 229-253 and 271-288 and two moderately hydrophobic peaks at position 131-148 and 185-210 (23). Currently two topological models for human bestrophin have been proposed, one with four transmembrane domains (23) and one with six transmembrane domains (18), the difference between them coming from considering the moderately hydrophobic peaks as integral transmembrane domains. Both N and C termini are located inside the cell. The model depicted in Figure 4.1 represents the most accepted topology of bestrophin, based on the hydropathical plot. In order to function as a chloride channel, bestrophin likely forms multimeric proteins (18;20) capable of heterooligomerizetion to produce a variety of ion channels.

Genomic studies identified three other homologues for human bestrophin and alignment analyses showed that the N-terminus is highly conserved among bestrophin homologues. Most of the variations among isoforms arise from the C-terminus (18). An interesting resemblance exists between the M2 domain of the GABA (A) receptor α1 subunit and the bestrophin RFP domain, located on the fourth transmembrane domain. This sequence is highly conserved among bestrophins family and is thought to play an important role in the forming of the channel pore (17).

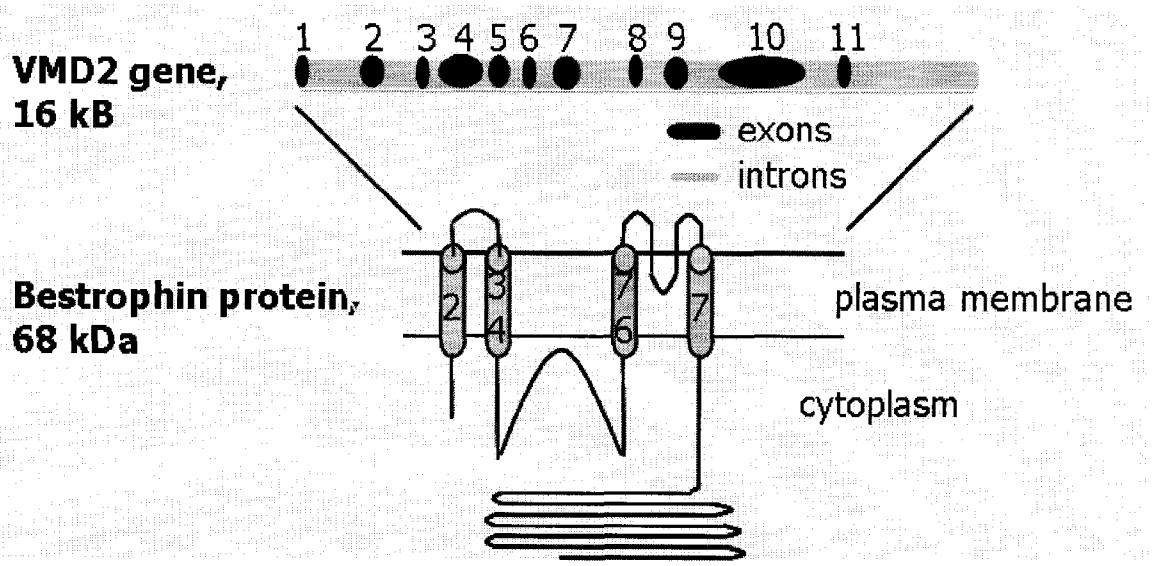

Figure 4.1 - Structure of bestrophin protein, the product of VMD2 gene. *VMD2 gene comprises of 11 exons and its product, bestrophin protein, has a molecular weight of 68 kDa and four transmembrane domains (the exons that encode the transmembrane domains are indicated), with both N- and C-termini located inside the cytoplasm.*

3. Expression and activation

Initially, when the VMD2 gene was identified, bestrophin was shown to be abundantly and specifically expressed in retinal pigment epithelium (15). Later, RT-PCR analysis of human isoforms showed a larger tissue distribution of the bestrophin homologues transcripts (19). Recent studies revealed that bestrophin is expressed and functions as a chloride channel in olfactory (16), airway, colon and kidney epithelial cells (2) .

Our laboratory demonstrated for the first time expression of bestrophin protein in human airway epithelial cells (5). Furthermore, immunohistochemistry studies on sections of human lung showed expression of bestrophin in airway smooth muscle, endothelium, pulmonary blood vessels and alveolar macrophages. Interestingly, serous cells, and not mucous cells, from airway submucosal glands demonstrate expression of bestrophin. Epithelial cells lining the lung alveoli (pneumocytes type I and II) displayed negative staining for bestrophin (Figure 4.2)

Expression of the bestrophin homologues is broadly distributed in nature. Members of the bestrophin family were identified in eukaryotes: twenty-six in *Caenorhabditis elegans*, four in *Drosophila melanogaster* and in mammals (17), and another four in fungi. Recently, prokaryotic homologues were found in certain Gram negative bacteria, but not in Gram-positive bacteria (7). Interestingly, bestrophin homologues haven't been identified in plants, yeast or protozoan.

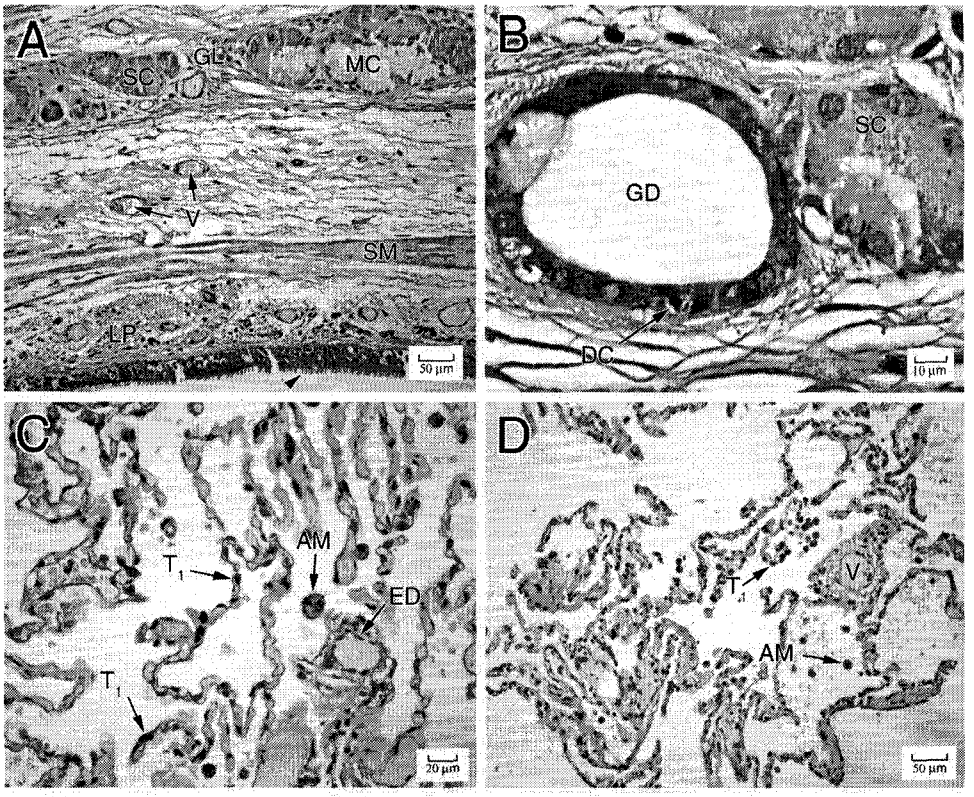

Figure 4.2 - Expression of bestrophin in human lung. *Immuno-peroxidase staining of formalin-fixed paraffin embedded lung tissue using monoclonal antibody to bestrophin. A, B, C – positive staining; D – isotype control, A – segmental bronchus (X 200) GL – submucosal glands, SM – smooth muscle cells, V – blood vessels, LP – lamina propria, SC – serous cells of submucosal glands, MC – mucosal cells of submucosal glands, arrow head pointing ciliated surface epithelium; B – submucosal gland (X 1000) GD – glandular duct, DC – ductal cells, SC – serous cells of submucosal gland; C – alveolar parenchyma (X 400) AM – alveolar macrophage, ED – endothelial cells, T_1 – type I pneumocytes; D – alveolar parenchyma (X 200), AM – alveolar macrophage, V – blood vessel, T_1 – type I pneumocyte*

Bestrophin interacts physically and functionally with protein phosphatase 2A (13). Consensus motifs for PKG and PKA were identified on the C-terminus, a highly regulated domain of the protein (5;18). In retinal pigment epithelium, bestrophin was found to be phosphorylated under basal conditions (13), while in airway epithelium phosphorylation is increased after treatment with nitric oxide donors (Duta et al., Journal of Membrane Biology, in press). Whether protein phosphorylation plays a role in channel activation remains to be demonstrated by future experiments

4. Physiological functions

Macular vitelliform dystrophy is functionally characterized by a decreased slow light peak in the electro-oculogram. The slow light peak of the electro-oculogram is thought to be due to depolarization of the basolateral membrane of retinal pigment epithelium as a consequence of an increase of the basolateral Cl^- conductance (6). The role of bestrophin in the eye is very well documented and different mutations of bestrophin were shown to be responsible for Best's disease (1). The majority of these mutations are on the N-terminus of the protein, suggesting that this part of the protein is important for the formation of the channel pore (8).

Arguments for bestrophin being a chloride channel are provided by several studies which indicate that, 1) different expression of bestrophin homologues in a variety of heterologous systems determined a chloride conductance (23), 2) bestrophin-induced currents are sensitive to classic chloride channel blockers, such

as DIDS, SITS and niflumic acid (2;5;16;22;23), 3) mutations in bestrophin produced modifications in chloride conductance, 4) bestrophin is a plasma membrane protein and silencing it through specific siRNA reduces the endogenous currents (2;4;5), 5) wide expression of bestrophin in different organisms and especially in prokaryotes suggests a role of this protein in adaptation to osmotic and pH stress. Recent studies revealed that bestrophin has a conductance of 2 pS, with a linear I-V relationship (4;16).

Two studies have demonstrated localization of bestrophin on the basolateral membrane in retinal pigment epithelium (12) and airway epithelium (5). Expression of Cl^- channels on the serosal side of epithelium has also been noted in kidney and intestine epithelium (3;11). The role played by the basolateral Cl^- channels in airway transepithelial anion secretion is especially important for diseases characterized by an impaired mucocilliary clearance. While CFTR is the main Cl^- channel on the luminal side of airway epithelium, presently there are several candidates for the serosal side: outwardly rectifying chloride channels, bestrophin, CLC-2 and CLC-K. Basolateral Cl^- channels in the human airway epithelium were suggested to act as molecular switch between Cl^- and HCO_3^- secretion (21), whereas in the intestinal mucosa and kidney basolateral Cl^- channels seem to be involved in NaCl and water absorption (3;11). Although CLC-K channels are known to be mutated in Bartter's syndrome, characterized by dehydration and kidney failure, CLC-2 channels are broadly expressed and no disease was assigned to CLC-2 mutants (10). In serous cells of the airway submucosal glands the basolateral Cl^- conductance is sensitive to micromolar concentration of DIDS (5;21), while

heterologously expressed CLC-2 are not sensitive to this extensively used chloride channel blocker (10). The presence of outwardly rectifying chloride channels on the basolateral membrane of airway epithelium has been demonstrated (9;21), but their molecular identity is presently unknown.

According to studies done in Calu-3 cells, an extensively used model of the serous cells from human airway submucosal glands we propose a new role for bestrophin. In these cells a Na^+/K^+ pump on the basolateral membrane maintains the physiological gradients of Na^+ and K^+. K^+ channels are located on the same side and have a role in recirculation of K^+ ions brought inside the cells by the pump. HCO_3^- enters the cells via a basolateral Na^+-HCO_3^- cotransporter (NBC) and exits via apical CFTR. Cl^- that is introduced by the Na^+-K^+-$2Cl^-$ cotransporter (NKCC) is translocated to the apical side where it exits via CFTR. Water is transported via an intercellular pathway, following NaCl secretion (Figure 4.3). The role of bestrophin in this model would be to recycle part of the Cl^- through the basolateral membrane. As was suggested by experiments from our laboratory, basolateral Cl^- channels are most likely closed or partly active under baseline conditions and fully open under inflammatory conditions, being regulated by markers of inflammation such as adenosine or nitric oxide. Therefore, we consider these channels important players in inflammatory airway diseases characterized by an impaired hydration of the hypersecreted mucus.

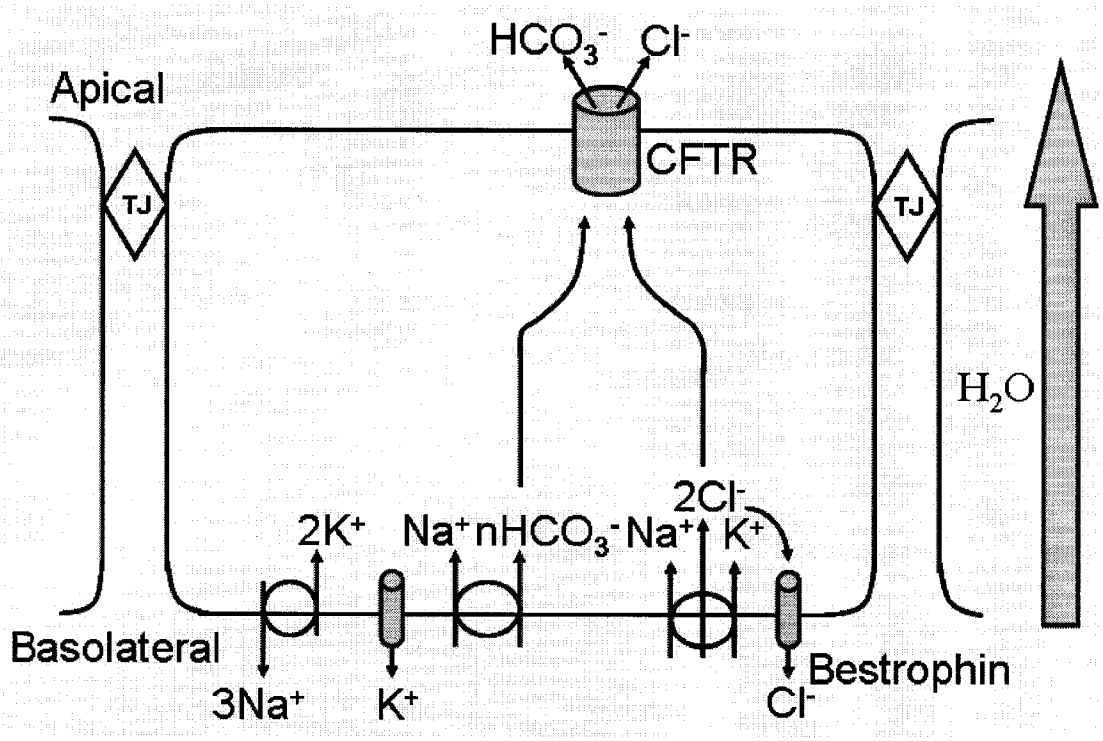

Figure 4. 3 – Anions transport model in serous cells from airway submucosal glands. *In polarized epithelial cells from submucosal glands there are Na^+/K^+ pumps, Na^+/HCO_3^- and $Na^+/K^+/Cl^-$ cotransporters assure proper anion entry, while on the apical membrane CFTR regulates Cl^- and HCO_3^- secretion. The role of basolateral bestrophin in these cells is to recycle part of the chloride that enters via $Na^+/K^+/Cl$ cotransporter.*

In RPE cells ion transport follows a similar pattern as in Calu-3 cells, the only difference being that the polarity is reversed, by having the NKCC, NBC and Na^+/K^+ pump on the apical membrane and Cl^- channels mainly on the basolateral membrane (6). RPE cells modify the direction of ion fluxes according to their physiological needs, being absorptive in the light and secretory in the dark. As in Calu-3 cells, Cl^- channels are present in both apical and basolateral membranes and bestrophin seems to be concentrated more on the basolateral membrane (12).

5. Putative target in different disorders

Cystic fibrosis is a channelopathy characterized by different classes of mutations of CFTR. Class I is characterized by an unstable mRNA and complete absence of CFTR, class II is a trafficking defect of the ΔF508 CFTR, in which 99% percent of the mutated CFTR is retained in the ER and does not reach the plasma membrane, class III produces an inactive CFTR which reaches the plasma membrane, class IV has a functional but low-conductance CFTR on the plasma membrane, and class V has reduced amounts of normal CFTR on the plasma membrane. CFTR was shown to conduct not only chloride, but also bicarbonate; therefore it presumably has a role in regulating the pH in human airways.

Given the proposed role of bestrophin in airway epithelium, it would be interesting to study transepithelial ion secretion in patients with Best's disease and investigate whether there is an increase in secretion of bicarbonate in the airways. In the same line, a patient with cystic fibrosis and a mutated bestrophin might have a more favorable outcome than a patient with only a mutated CFTR. Since patients

with cystic fibrosis have a variety of phenotypes, we propose the study of bestrophin expression in airway epithelium of these patients, especially for the ones with a class IV or V mutations.

The role of bestrophin in chloride recycling across the basolateral membrane of airway epithelium makes this molecule a putative target in airway diseases characterized by inflammation, such as asthma or chronic obstructive pulmonary disease. Under these conditions, a chloride channel blocker targeting bestrophin would be beneficial, since the focus of the treatment would be to maintain salt and water secretion for a proper hydration of the hyper secreted mucus which characterizes these airway disorders.

In Best's macular dystrophy, gene therapy could be attempted in order to restore the Cl^- flux through the basolateral membrane of RPE. A chloride channel opener could also be tested, especially at early stages of the disease, although experiments with mutated bestrophin and chloride channel openers have not been designed yet.

Bestrophin has been found to be expressed also in non-epithelial tissues, such as smooth muscles, endothelium or alveolar macrophages. The exact contribution of bestrophin to the physiological processes occurring at these sites remains to be addressed by future studies.

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CHAPTER 5

General discussion

Earlier studies have shown that NO stimulates CFTR and potassium channels in airway epithelial cells (7;8). My studies expanded these observations by showing that NO plays a crucial role in regulation of basolateral chloride channels. The main results of this research project were that the bestrophin chloride channel is a molecular target for NO in airway epithelium, and the characterization of the signal transduction pathway through which basolateral chloride channels are regulated by NO.

We used Calu-3 cells as experimental model for several reasons: 1) they constitute a model for human serous submucosal gland epithelium, expressing high levels of CFTR and other markers of submucosal epithelium, including lysozyme, lactoferrin and secretory leukocyte protease inhibitor (11;25); 2) when grown as monolayers, they form tight junctions with moderate transepithelial resistance, allowing the measurement of I_{sc} in Ussing chambers (25); 3) they allow the study of NO effects on ion channels. Moreover, they likely express PKGII (4;7); 4) they constitute an excellent model to study basolateral Cl^- channels in comparison with other cell lines, because they have a higher level of activity of these channels than for example, normal human bronchial epithelial (NHBE) cells (a cell line used as a model for columnar airway epithelium) (28); 5) they constitute a widely accepted model of airway epithelium (13;14).

The presence of basolateral chloride channels in airway epithelial cells was demonstrated almost 2 decades ago (31). After disruption of the monolayers, Calu-3 cells showed an outwardly rectifying depolarization induced Cl^- channel (ORDIC), but this channel was not found in the apical membrane of confluent monolayers (32).

In a more recent study, basolateral Cl^- channels were shown to be DIDS-sensitive and to be involved in chloride absorption (15). However, our lab has proposed that these channels play a role in recycling chloride across the basolateral membrane of Calu-3 cells and in preferential secretion of HCO_3^- over Cl^- by Calu-3 cells (28). One important difference that might explain discrepancies in these studies was the concentration of DIDS used, our lab using a four fold smaller amount of DIDS than Hwang's group (15).

The hypothesis that basolateral chloride channels stimulate HCO_3^- secretion was based on the analogy between basolateral potassium and chloride channels. Activation of basolateral potassium channels leads to hyperpolarization of the cell, followed by a stimulation of the Cl^- secretion; furthermore, it inhibits the activity of the Na^+-HCO_3^- cotransporter, allowing preferential secretion of Cl^- over HCO_3^- (29). In a similar manner, stimulation of basolateral chloride channels depolarizes the cell, stimulates Na^+-HCO_3^- cotransporter and HCO_3^- secretion; therefore, these channels could act as a molecular switch between Cl^- and HCO_3^- secretion (28).

The lack of specific chloride channel blockers was one of the major obstacles that we had to overcome in our experiments. Basolateral chloride channels are DIDS sensitive (15;32), but DIDS is not a specific blocker of these channels. DIDS can block also the Na^+-HCO_3^- cotransporter and Cl^-/HCO_3^- exchanger, although the importance of the latter is not significant, given that it is electroneutral. In the intestinal cell line T84, DIDS was suggested to stimulate chloride secretion by elevating intracellular Ca^{2+} and activating apical calcium-dependent chloride channels (2). However, a direct measurement of Ca^{2+} concentration after DIDS

addition was not possible and there was a discrepancy noticed between the effects of DIDS in patch clamp experiments versus transepithelial measurements. Furthermore, the same group showed that the apical membrane of Calu-3 cells is DIDS insensitive either by application of DIDS on an intact monolayer or after permeabilization of the basolateral membrane (21). DIDS is impermeable through plasma membrane, so its effects are likely to be exerted through the extracellular domains of a channel or transporter (23). CFTR can also be blocked by DIDS, but only when applied from the intracellular side (17). Altogether, these findings suggest that DIDS effects on the basolateral side of Calu-3 cells are due to inhibition of basolateral chloride channels and Na^+-HCO_3^- cotransporters. These findings are also based on the experimental observation that in the presence of GSNO in HCO_3^- free Krebs Henseleit solution (KHS) basolateral addition of DIDS causes an increase in I_{sc}, while in Cl^- free KHS a decrease in I_{sc}. This latter effect is most likely due to the blocking of the Na^+/HCO_3^- cotransporter.

One of my goals was to identify the basolateral chloride channels at the molecular level. In 2002, bestrophin was suggested to form a new class of ion channels (27). Based on its sensitivity to DIDS, its localization to the basolateral membrane of retinal pigment epithelium and selectivity (20;27) we hypothesized that this channel may play a role in the basolateral chloride conductance in Calu-3 cells. To test our hypothesis we employed classical approaches, RT-PCR and Western blotting to show the expression of bestrophin in Calu-3 cells. Having this established, we employed RNA interference technique to show for the first time the role of bestrophin in airway epithelial ion transport by reducing its endogenous expression (9). Since its discovery

in 1998, RNA interference gained more and more appreciation from researchers for its efficiency and specificity in protein knock down; therefore, in 2006, the inventors of this powerful research tool were awarded the Nobel Prize in Medicine and Physiology (6;12).

Four bestrophin isoforms are currently known and all of them conduct Cl^- when expressed in HEK 293 cells. Bestrophin isoforms display approximately 65% homology, with N-termini almost identical, but with different C-termini (19). Among all bestrophins, bestrophin 4 exhibits the largest current, which is Ca^{2+} dependent (30). When we designed the siRNA duplexes, we chose a region common to all isoforms, so we were unable to have conclusive information on which isoforms are involved in the basolateral chloride conductance in Calu-3 cells (9). To study basolateral chloride channels we used transepithelial measurements of I_{sc} in Ussing chambers, a technique that can isolate the contribution of the basolateral membrane. A recent study demonstrated that bestrophin 1 and 4 are expressed in Calu-3 cells and that they can be blocked by DIDS and activated by Ca^{2+} or ATP (1). These authors employed patch clamping and RNA interference against bestrophin 1 along with a specific antibody for bestrophin to confirm the efficiency of siRNA duplexes. The results of these experiments not only confirmed our hypothesis about the role played by bestrophin in Calu-3 cells, but also reproduced our experiments with a complementary technique.

Although our studies indicate that bestrophin likely plays a major role in basolateral chloride conductance in Calu-3 cells, we do not exclude a possibility that other chloride channels could also be involved. Calu-3 cells have been shown to

express non-CFTR chloride channels, including CLC-2 and CLC-K (22). CLC-2 appears to localize on the basolateral membrane of the intestinal epithelium (3;33) and CLC-K on the same side of kidney epithelium (18). Since CLC-2 channels are sensitive to $ZnCl_2$ blockage, we showed that these channels are likely to be localized on the basolateral membrane of Calu-3 cells, but they do not play a role in the GSNO stimulated basolateral chloride conductance. However, the precise contribution of every channel to the basolateral chloride conductance is difficult to assess, given the lack of specificity of chloride channel blockers. Furthermore, although siRNA is more powerful than antisense oligonucleotides (12), different duplexes designed against bestrophins in several cell models were unable to achieve a silencing effect greater than 70% (1;5;9).

Basolateral chloride channels have been previously shown to be activated by adenosine via a cAMP-dependent pathway (28). I chose to study the regulation of these channels by NO for several reasons: 1) NO is an activator of apical CFTR and basolateral potassium channels in Calu-3 cells (4;7); 2) NO and GSNO are present in human airways, and decreased amounts of NO in exhaled air were reported in CF patients (10;16); 3) GSNO has beneficial effects in CF (24;26).

When applied in Ussing chambers, GSNO produces a biphasic response: initial rapid (<1 min) increase in the I_{sc}, followed by a decrease to control levels (~ 4 min). We suggest that the lack of sustained effect on I_{sc} after addition of GSNO is due to the activation of basolateral chloride channels. Our hypothesis is based also on the fact that bestrophins display delayed activation and inactivation in patch clamp experiments (30). NO can activate ion channels via a cGMP dependent or

independent mechanism (8). However, based on the effects of the inhibitors of soluble adenylyl cyclase and PKG, ODQ and KT5823, respectively, on basolateral chloride channels, we suggest that these channels are regulated by NO mainly via a cGMP-dependent mechanism.

Lipid rafts are plasma membrane microdomains, involved in regulation of different signaling processes and characterized by an increased content of cholesterol. Since basolateral Cl^- channels are highly regulated proteins, particularly by inflammatory mediators, such as adenosine and NO, we hypothesized that they might reside in lipid rafts. In yet unpublished studies, I_{sc} measurements showed that the removal of cholesterol by basolateral methyl-beta-cyclodextrin (MβCD) blocked the response to DIDS after GSNO, indicating that basolateral Cl^- channels reside in lipid rafts. Co- immunoprecipitation and confocal microscopy studies demonstrated that bestrophin physically interacts with caveolin, a marker of lipid rafts. Moreover, pretreatment with short-interference RNA designed against caveolin decreased I_{sc} response to basolateral DIDS, in the presence of GSNO. Overall, our results suggest that airway epithelial cells could regulate anion secretion via modifying content of plasma membrane cholesterol and affecting the integrity of lipid rafts.

Conclusions

We have investigated the role of NO in the regulation of basolateral chloride channels in airway epithelium. Although NO is present in the human airways and it has been studied for long time, its precise physiological functions have not been established. The high levels of NO released by iNOS prevent viral replication and aid in elimination of various pathogens. NO is also known to regulate ion channel activity in the airway epithelium, contributing to the regulation of the ASL. Derangements in composition and quantity of ASL lead to improper cilia beating and impaired mucociliary clearance. Based on the effect of NO on the basolateral chloride channels and ASL, we proposed a new mechanism through which NO contributes to the innate defense in the human airways.

Basolateral chloride channels have been studied for almost two decades, but little is known about their molecular identity. We propose that bestrophins, a new family of chloride channels, play a major role in the airway epithelium basolateral chloride conductance. CLC-2, which has also been demonstrated to reside in the basolateral membrane of intestinal epithelium, may be involved, but our experiments showed that it is not likely to be stimulated by NO.

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