MOLECULAR CHARACTERIZATION OF AN AIRWAY CHLORIDE CHANNEL

Background and Significance

Large airways are more than conduits for air flow to sites of gas exchange in the lungs. Epithelia lining the airway surfaces, from the nose to the alveoli, form the first line of defense against inhaled irritants. The epithelium's response to noxious particulate and chemical stimuli is a critical component in the development of many lung diseases.

Mucociliary clearance is the process that removes inhaled particulates from the lungs. As a defense mechanism, it protects against infection, reduces cancer and disease risks from inhaled carcinogens and toxins, and prevents airway obstruction. Ciliated epithelial cells propel a nearly continuous layer of mucus out of the lungs. Optimal efficiency depends on the hydration state of airway mucus and the depth of the fluid layer surrounding cilia. These, in turn, depend on the coordinated regulation of epithelial fluid secretion and absorption.

Acute exposure to cigarette smoke inhibits epithelial chloride secretion with little or no effect on the rate of sodium absorption. The combined effect is an increase in net fluid absorption. Under this condition, the viscosity of airway mucus will increase and the efficiency of mucociliary clearance should decrease correspondingly. Indeed, cigarette smoke has long been known to impair mucociliary clearance. Prolonged exposure to cigarette smoke causes loss of ciliated epithelia and increased epithelia permeability, both of which could result from the acute inhibition of chloride secretion. Thus, minimizing the effect of cigarette smoke on airway chloride secretion could reduce its more long term negative effects on ciliated cells and epithelial barrier properties.

The observation that chloride secretion is reduced without an effect on sodium transport suggests that components of cigarette smoke affect directly the activity of secretory chloride channels. Therefore, it is critical that this channel be identified and its regulation studied. The chloride channel or channels that mediate the bulk of airway chloride secretion have not been defined at the molecular level. The defective gene in cystic fibrosis was thought to hold the key. However, even though the CFTR protein functions as a chloride channel in some tissues, the level of its expression in the lungs is too low to mediate more than a fraction of total airway chloride secretion. It now appears that in the lungs, the function of CFTR is to regulate additional chloride channels and perhaps sodium channels as well. The molecular nature of these additional channels is completely unknown.

Broad Goals and Specific Aims

The long range goal of this project is to clone and sequence an airway chloride channel. A method has been developed to isolate intracellular membrane vesicles enriched in chloride channels from airway (bovine trachea) and other epithelial tissues. This method is based on hydrophobic affinity chromatography and yields a membrane vesicle fraction that is enriched in chloride channels 30 to 40 fold over unfractionated tissue homogenates. This project has the following specific aims based on this channel purification method:

SPECIFIC AIM #1 To use hydrophobic chromatography to partially purify an airway chloride channel and to use this as starting material for the development of polyclonal and monoclonal antibodies against the chloride channel and associated proteins.

SPECIFIC AIM #2 Candidate channel proteins will be identified based on their biochemical and functional properties in several reconstitution assays and microsequenced. Oligonucleotide probes based on these sequences will be used to screen tracheal cDNA libraries for positive clones, which will then be sequenced.

Together, the antibodies and cDNA probes generated in this project will be important tools for studying the molecular and cell biology of this airway chloride channel and the biochemistry of its regulation.

Preliminary Studies

A hydrophobic affinity chromatography method has been developed to isolate intracellular membrane vesicles enriched in chloride channels from airway (bovine trachea) and other epithelial tissues.This method yields a vesicle fraction enriched 30 to 40 fold in chloride channels over unfractionated tissue homogenates. This vesicle fraction contains a single type of chloride channel, which is also present in apical membrane fractions from the same tissues. The vesicle chloride channel exhibits a unique profile of biophysical properties when studied in artificial bilayers (70 pS linear conductance in symmetric salt solutions, $Cl > Br \geq I$ selectivity, inhibition by the anion channel blocker DIDS). These properties differentiate this channel from the CFTR chloride channel. The intracellular membrane vesicles containing this channel lack marker enzymes commonly used to identify intracellular organelles. However, our very recent immunological assays suggest that these vesicles originate from the trans-Golgi network (TGN). I hypothesize that these vesicles represent a transport intermediate that shuttles secretory chloride channels between the apical plasma membrane and an intracellular storage organelle.

The chloride channel-enriched vesicles from bovine trachea contain relatively few proteins. There are 10-12 proteins visible in Coomassie blue-stained SDS-PAGE gels with major proteins bands at 200, 70, 45 and 30-35 kDa. Some of these proteins are found in chloride channel-enriched vesicles isolated from bovine and rat kidney and a human colonic cell line (T84). Vesicles from these sources contain the same chloride channel as the tracheal vesicles. One of the common proteins present in the vesicles from all of these sources may be the airway secretory chloride channel.

My recent immunological characterization of the vesicular 200 kDa protein (p200) demonstrates that it is a newly described trans-Golgi network (TGN) protein. Work by other groups has shown that the p200 is a novel coat protein for TGN-derived membrane vesicles. Others have shown that most of the cellular content of p200 is soluble. However, I have shown that the vesicular p200 is insoluble in mild detergents and preliminary evidence suggest that it exists in macromolecular complex with the major 45 kDa protein and other less abundant proteins. In addition, preliminary data suggests that p200 or other vesicle proteins interact with a calcium and phospholipid binding protein of the annexin falmily. Some annexins are known to regulate epithelia chloride channel activity.

Experimental Design and Procedures

Previous work in my laboratory has used bovine trachea as the main source of tissue for biochemical studies of airway chloride channels. However, for this project human airway cell lines (Calu3 and 9HTEo-) will be used as starting material. Any of the molecular and immunologic reagents developed in this project can then be used to study the cellular regulation of airway ion transport in these cell lines.

Hydrophobic affinity chromatography will be used to isolate chloride channel-enriched vesicle from these cells. A ^{36}Cl flux assay will be used to follow channel purification. Highly enriched vesicle preparation will be used to generate monoclonal antibodies. Purified vesicles, emulsified in Freund's adjuvant, will be used to immunize mice. When test bleeds indicate high titers of antibodies, spleens will be removed, lymphocytes isolated and fused with lymphoma cells according to established protocols. Hybridoma supernatants will be screened for channel and vesicle-specific reactive antibodies in two ways. First, supernatant will be screened for reactivity against vesicle proteins by immunobloting. Second, supernatants will be screened for chloride channel-inhibiting activity using either the ^{36}Cl flux assay or in an electrophysiological assay after reconstituting the vesicular chloride channel into planar lipid bilayers. In addition, polyclonal antisera to the major vesicular proteins (e.g. 200, 70, and 45 kDa) will be developed in rabbits.

I have begun to characterize the detergent extraction properties of the major vesicle proteins. This approach will define which vesicle proteins are integral to the membrane, as expected for a channel protein. Additional chemical crosslinking studies will define some of the molecular interactions among vesicular proteins. Also, vesicle proteins will be solubilized, fractionated and reconstituted into artificial lipid vesicles and assayed for channel activity by ^{36}Cl flux. Using these and other biochemical approaches to characterize the vesicle proteins, candidate chloride channel proteins will emerge. These proteins will be partially purified by conventional methods (ion-exchange and gel filtration chromatography, HPLC) and separated by 1D and /or 2D gel electrophoresis. Single gel bands or spots will be microsequenced by a commercial service. To facilitate sequencing of proteins with blocked amino termini, partial proteolytic digestion with cyanogen bromide or TPCK-treated trypsin will be used. Based on these partial amino acid sequences, oligonucleotides will be synthesized commercially and used to probe a human airway cDNA library (also available commercially). Positive clones will be sequenced and used to reprobe the library in an effort to generate full coding length clones or overlapping clones that can be pieced together into a full length cDNA.

The immunological and molecular reagents developed in this project will provide significant insight into the molecular identity of this airway chloride channel and the biochemistry of its regulation. Also, they will be used to develop future projects to probe the tissue-specific and developmental expression patterns of this channel, its role in airway cell biology, ion transport regulation, and the pathophysiology of lung disease, and structure/function relationships within the channel protein and between the channel protein and other proteins in these isolated vesicles.