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Chloride transport by tracheal epithelium

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CHLORIDE TRANSPORT BY TRACHEAL EPITHELIUM
by

Peying Fong

DISSERTATION

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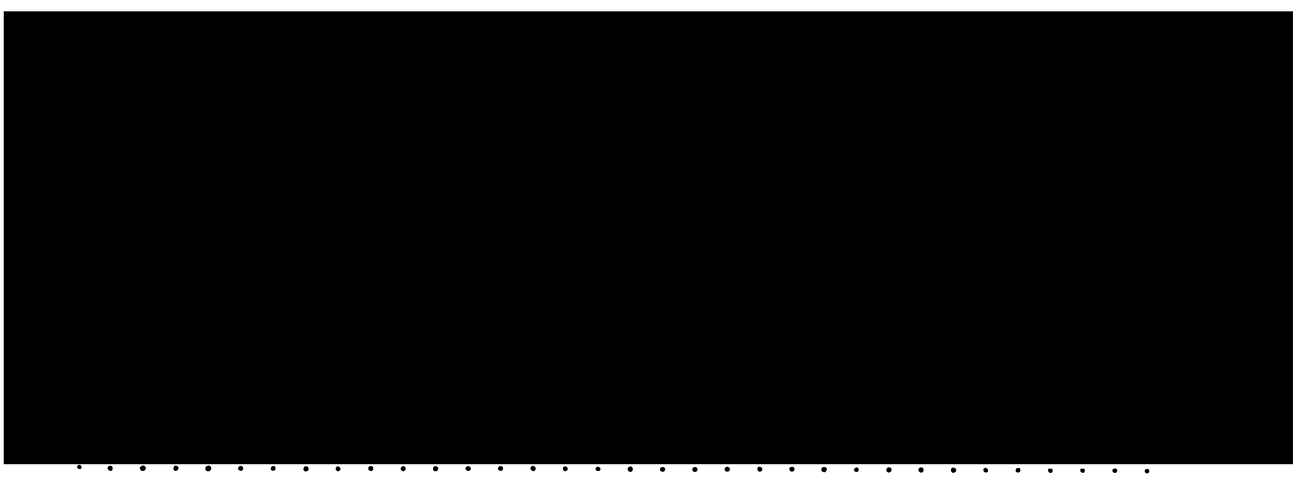

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CHLORIDE TRANSPORT BY TRACHEAL EPITHELIUM

Peying Fong

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August 10, 1989

To Whom it May Concern:

The great part of chapter 2 of this thesis is reproduced from "Chloride Transport in Apical Membrane Vesicles from Bovine Tracheal Epithelium: Characterization Using a Fluorescent Indicator", by P. Fong, N.P. Illsley, J.H. Widdicombe and A.S. Verkman, which appeared in the Journal of Membrane Biology, volume 104, pages 233 to 239, 1988. All of the experiments described in this article were performed by Peying Fong, and she was primarily responsible for writing the article. Drs. Illsley, Widdicombe and Verkman supervised various aspects of the work. The work performed was of exactly the same kind as, and comparable in scope and contribution to, that involved in unpublished material in this and other theses/dissertations.

Jonathan Widdicombe
Associate Professor of Physiology
Research Advisor

Dedication

This dissertation is dedicated to my many teachers.

"The highest function of the teacher
is not so much in imparting knowledge
as in stimulating the pupil
in its love and pursuit."

—Amiel

Table of Contents

List of Figures

List of Tables

Chloride Transport by Tracheal Epithelium

Peying Fong

Abstract

Basic mechanisms underlying Cl secretion by tracheal epithelium have been examined in the following studies. The vectorial movement of Cl from the serosa to the mucosal aspect of the tissue consists of two processes: 1) uptake of Cl from the blood across the basolateral membrane and 2) its exit from the cell through a conductive Cl-selective apical pathway. The transporters involved in this mechanism were studied in detail using several different techniques.

The apical Cl conductive pathway was first considered. Apical membrane vesicles were purified from bovine tracheal epithelium and Cl influx was monitored using the halide sensitive fluorescent indicator 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ). This fluorescence assay, with its intrinsically high level of resolution, rendered it possible to assess quantitatively the basic biophysical properties of the native apical membrane Cl transporter. The results of this study support the presence of a pathway for Cl transport that is 1) conductive 2) sensitive to diphenylamine 2-carboxylic acid 3) saturable and 4) an aqueous pore. Moreover, in these membranes there is no contribution by Na-coupled Cl cotransport or Cl/OH exchange to Cl transport.

Regulation of apical membrane Cl transport by two different systems was examined. Putative macromolecular regulators of Cl transport were entrapped in bovine tracheal apical membrane vesicles by hypotonic lysis and conductive Cl transport was assayed using the SPQ fluorescence quench technique. Entrapment of 1) the catalytic subunit of the protein kinase A, in the presence of ATP,

and 2) GTP-γ-S, in the presence of Mg^{++}, had no effect on the initial rate of Cl influx.

The basolateral Cl uptake mechanism was examined in primary cultured monolayers of canine tracheal epithelial cells using two complementary approaches. Both short circuit current (I_{sc}) and radioactive tracer uptake studies provide evidence for the presence of a K-dependent, Na-coupled Cl cotransporter in the basolateral membrane.

These studies support a model for tracheal Cl secretion in which 1) Cl is taken up at the basolateral membrane by a K-dependent, Na-coupled Cl transporter and 2) exits across the apical membrane exclusively via a Cl conductance. Regulation by protein kinase A and direct G protein interactions, if involved in this system, must require cellular components not found in the purified apical membranes used in this study.

Chapter One

Introduction

Fluid transport by epithelial tissues results from the transepithelial movement of electrolytes. The tracheal epithelium normally maintains a thin electrolytic fluid layer upon which airway mucous secretions rest. This fluid layer thus provides a medium in which the cilia of tracheal epithelial cells can beat, and their tips contacting the underside of the mucous layer facilitate the flow of mucus toward the throat. Airway fluid levels are determined by two ion transport processes: Na absorption and Cl secretion. Sodium absorption results in the movement of salt, and hence, water, from the mucosa to the serosa of the tissue. Thus, the net effect of this process is to lower the fluid level. The need to raise fluid levels normally induces Cl secretion; net movement of salt and water from the blood to the luminal aspect of the tissue results. The exact levels of airway fluid must be regulated within a very narrow range for proper functioning of the mucociliary clearance mechanism. Thus, the balance of these two ion transport processes is critical to the physiology of the tissue.

In the genetic disease, cystic fibrosis, alterations in airway ion transport lead to mucous secretions that are unusually viscous. This hinders the normal clearance of mucus from the airways, as cilia cannot generate adequate force to propel this thick secretion. However, before the malfunction in cystic fibrosis can be completely characterized, the normal mechanisms of vectorial transport must be established clearly.

Historical Overview

Development of the Model for Tracheal Ion Transport

The net movement of NaCl across an epithelium has been studied using a variety of techniques. Figure 1 summarizes the currently accepted model of tracheal salt transport. Briefly, intracellular Na levels are kept low, with the expenditure of ATP, by the basolaterally situated Na/K pump. The resulting inwardly-directed Na gradient fuels two ion transport processes: Na absorption and Cl secretion. In the case of Na absorption, Na enters across the apical membrane, via a Na channel, and is then extruded into the serosal milieu by the action of the ATPase. Under certain conditions (i.e. β agonist stimulation), Cl secretion is elicited. Here, Cl enters the cell from the blood via a Na-coupled mechanism and accumulates to a level above that expected from the electrochemical forces across the apical membrane. Net passive exit of Cl across the apical membrane, through an apical membrane Cl conductance, follows and results in the net secretion of Cl.

The evidence that Cl secretion proceeds via the mechanism described above was reviewed by Frizzell et al (1979) and is summarized as follows. Cl secretion by rabbit ileum and colon, frog gastric mucosa and cornea, and shark rectal gland was found to be dependent on the presence of basolateral Na; removal of Na inhibited Cl secretion (Nellans et al, 1974; Sachs et al, 1978; Silva et al, 1977; Candia, 1972; Zadunaisky, 1966 and 1972). In addition, the serosal application of furosemide, a "loop" diuretic inhibitor of NaCl cotransport, as well as ouabain, a well known blocker of the Na/K ATPase, abolished Cl secretion (Sachs et al, 1978; Silva et al, 1977; Candia, 1972; Zadunaisky and Spinowitz, 1977). In rabbit colon, while cAMP- or A23187-stimulated Cl secretion caused a large increase in

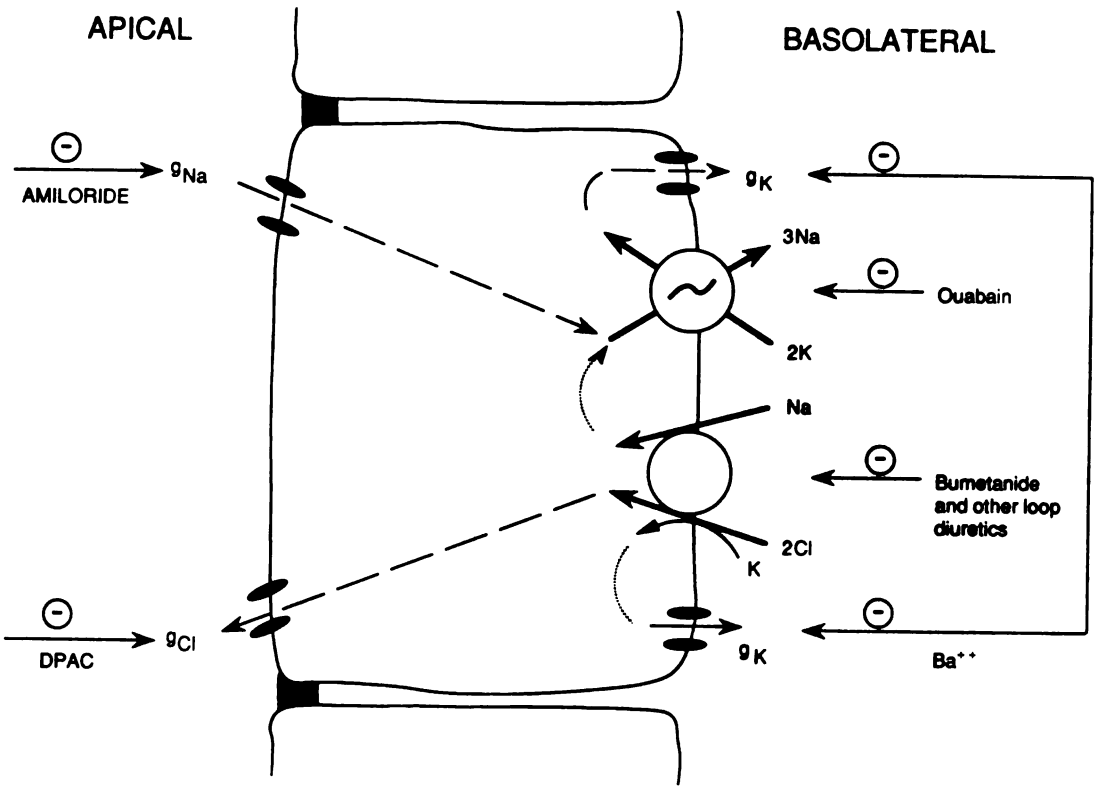

Figure 1. Model of tracheal electrolyte transport. See text for explanation. Adapted from Frizzell et al (1979).

tissue conductance, there were no changes in the unidirectional flux of Cl from mucosa to serosa or either unidirectional Na flux (Frizzell et al, 1976 and 1977). Thus, the secretion-associated conductance change could be ascribed to a decrease in the resistance of a Cl-selective transcellular component, rather than a change in the (relatively non- selective) paracellular pathway. In rabbit corneal epithelium, the site of such a decrease in resistance was localized to the apical membrane by the observations of Klyce and Wong (1977). Upon epinephrine-stimulation of Cl secretion, these workers measured a decrease in apical membrane resistance that was not observed when the tissue was bathed in Cl-free medium. Thus, the resistance change associated with secretion could be attributed to an increase in the Cl permeability of the apical membrane.

Characterization of Transepithelial Tracheal Ion Transport

In many initial characterization studies of ion transport, Ussing's short-circuit current technique (Ussing and Zerahn, 1951) has proven to be of great importance. In this method, the active transport of ions across a membrane can be measured as the short-circuit current (I_{sc}). The technique is based on the concept that active electrogenic ion transport—that is, ion transport resulting in generation of a transepithelial potential difference—causes equal currents via tissue shunt and/or leak pathways. Experimentally, this shunt current can be measured using an external circuit containing a Variac and an ammeter. The variable current source can be adjusted to pass current exactly offsetting the transepithelial p.d. to zero. The ammeter in the circuit monitors this current. Thus, the magnitude of this externally applied current is equal to that current actively generated by the tissue.

The sided effects of specific pharmacologic agents on the net movement of ions represented by the I_{sc} allowed the identification of the relevant transporting

moieties on both membranes of an epithelium [Welsh, 1987]. Thus, addition of amiloride, an inhibitor of epithelial Na channels, to the mucosal side of a tracheal epithelial sheet results in attenuation of the I_{sc}, whereas this maneuver is without effect when performed serosally. "Loop" diuretics such as furosemide and bumetanide, however, inhibit the isoproterenol-stimulated I_{sc} from the serosal, rather than mucosal, side of the tissue. Diphenylamine-2-carboxylic acid (DPAC), an anthracene compound known to block the Cl conductance in several epithelia, has no effect when applied serosally, but decreases I_{sc} from the apical side. Taken together, these observations support the model for tracheal salt transport depicted in figure 1, in which the two major transepithelial ion transport processes—Na absorption and Cl secretion—depend on the presence of apical Na and Cl conductive pathways, and basolateral Na-coupled Cl transport.

Microelectrode measurements support the predictions of a model based on the Ussing chamber data. Figure 2 illustrates these predictions. From the literature, the values for the intracellular Na, Cl, and K activities (a_{iNa}, a_{iCl}, a_{iK}; Shorofsky et al, 1984, 1986, Smith and Frizzell, 1984) can be obtained, as can the apical and basolateral membrane potentials (ψ_a and ψ_{bl}; Shorofsky et al, 1986). The absolute differences between the respective Nernst equilibrium potentials and the ψ_a or ψ_{bl} determine the net driving forces for transmembrane ion movements. Thus, it can be calculated that the net driving forces for the processes governing Cl secretion—basolateral Na uptake and K exit, and apical Cl exit, are 120, 12, and 28 mV, respectively. These electrophysiological approaches—microelectrode and I_{sc} measurements—have been carried out not only in intact epithelial tissues, but also primary cultured monolayers of tracheal epithelial cells, a preparation shown to be a good model for Cl secretion by the native tissue (Widdicombe, 1986b).

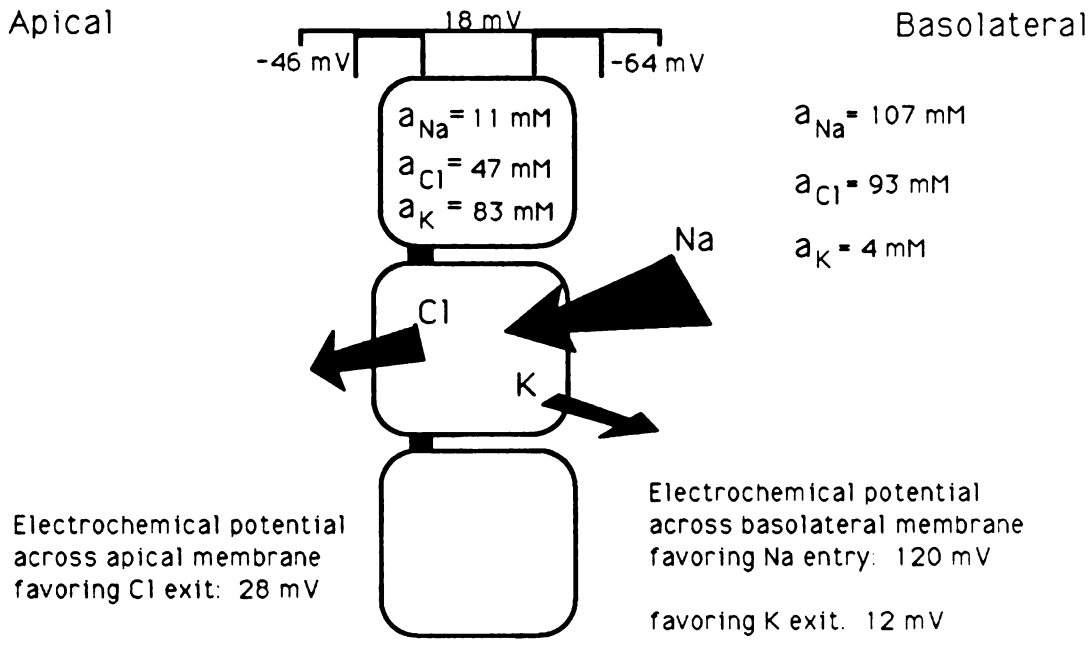

Figure 2. Driving forces determining net Cl transport. Details in text.

Studies of Transport Pathways in the Apical Membrane:
Characterization and Regulation

Langridge-Smith (1983) developed a method for the purification of apical membranes suitable for tracer flux studies from bovine tracheal epithelium. Thus, apical membrane transport could be followed in a system uncomplicated by basolaterally mediated ion movements. The results obtained by these investigators indicate the presence of conductive pathways for the uptake of both Na and Cl (Langridge-Smith et al, 1983, and 1984). Therefore, the major transport processes of the intact tracheal apical membrane—amiloride-sensitive Na channels and the Cl conductance—have been demonstrated to be preserved in membrane vesicles. Several basic properties of these mechanisms were not quantitatively assessed due to resolutary limitations of radioactive tracer measurements. Nevertheless, the data are consistent with the salient features of apical membrane transport presented in the model of figure 1.

More recently, studies from the laboratories of Welsh (1986 a and b) and Frizzell (1986b) have employed the technique of single channel recording (Hamill et al, 1981) to study the apical membrane Cl conductive pathway of tracheal epithelium. Thus, information such as the unitary conductance, rectification properties, and anion selectivity has been obtained via patch clamp technology. Moreover, these workers were able to utilize inside-out patch recording to study the regulation of the Cl conductance by intracellular second messengers such as cAMP and Ca^{++}. Welsh (1986) reported the properties of the Cl conductance in inside-out patches from canine tracheal epithelial cells in primary culture. The channel studied had a single channel conductance of 29 pS in symmetrical (145 mM Cl) solutions and was outwardly rectifying. Selectivity for Cl over the anions, SO_4 and gluconate, as well as cations, was demonstrated. Inhibition of

channel activity by DPAC was dose-dependent, and this relationship was virtually superimposable with that demonstrated for I_{sc} inhibition by the same compound. Thus, it can be inferred that this channel is responsible for the I_{sc} changes associated with Cl secretion. The mechanism of channel blockage by DPAC was not investigated extensively; although a current amplitude decrease with no effect on single channel kinetics was speculated upon, the possibility of rapid flicker block was not ruled out. Finally, the regulation of the channel by isoproterenol and Ca^{++} was examined. Activation of the channel by isoproterenol in the cell-attached mode occurred in 8 of 43 patches in which the channel was observed. In 4 cell-attached patches, the channel was spontaneously active in the absence of exogenous factors. The remaining 31 patches showed channel activity only after excision and formation of the inside-out configuration, suggesting that there may be an endogenous inhibitor of the Cl channel. Upon excision of the patch, this factor conceivably is washed away from the membrane, thus permitting channel activation to proceed. While these studies do not explicitly identify cAMP as the relevant second messenger, the possibility that this indeed is the case is strengthened by the previous studies of Al-Bazzaz (1981) which demonstrated stimulation of Cl secretion upon addition of cAMP to dog tracheal mucosa. Welsh also investigated the possibility of a role for elevated [Ca^{++}] in the regulation of Cl channels. In inside-out patches, raising [Ca^{++}] to 1 mM in the bath solution did not induce channel stimulation, thus arguing against a role for direct Ca^{++} regulation of the Cl conductance. These data are difficult to interpret because the probability of opening (P_o) for Cl channels in these excised patches was already very high. Similar findings were obtained on cells derived from human tracheal epithelium and reported in a later paper (Welsh, 1986). Hence,

the observations made in dog cells can be extended to include channels in human tracheal cells.

Frizzell et al (1986b) reported data suggesting that the inability of cystic fibrosis (CF) tracheal epithelium to secrete Cl rests in a defective regulation, not an absence or a lack, of Cl channels. This group demonstrated ß-adrenergic stimulation of 2 different Cl channels in normal human tracheal epithelial cells in the cell-attached configuration. Two conductance levels were observed: a non-rectifying 20 pS channel, and an outwardly rectifying 50 pS conductance similar to that described by Welsh. When the same studies were carried out on CF derived cells, no ß-adrenergic stimulation of channel activity was observed. However, excision of CF derived patches (inside-out) into the bath (Hepes-buffered Ringer solution) resulted in activation of Cl channels. Both conductance levels were observed and these had biophysical characteristics not discernable from those of normal cells.

Further investigation led to the conclusion that channel activation could result from a Ca^{++}-mediated event; channels opened by excision into a 500 nM free Ca^{++} solution could be inhibited upon addition of the Ca^{++} chelator, EGTA, to the bath. In on-cell patches, Cl channels in CF derived cells could be stimulated by application of A23187, a Ca^{++} ionophore. This observation is in agreement with the data of Widdicombe (1986a); in cultured confluent monolayers of CF tracheal cells that had Na absorption blocked by amiloride, I_{sc} was stimulated stepwise by cumulative doses of A23187. However, Welsh and Liedtke (1986) found Cl channel activation in CF cells after excision into Ca^{++}-free medium (< 1 nM) and interpreted this to mean that excision alone induces channel opening. Indeed, Welsh's hypothesis that normal cells possess a channel inhibitory factor that is washed away during patch

excision could be extended to explain these results—this inhibitory factor must still be present in CF tracheal epithelial cells. Thus, while these groups agreed on the basic biophysical properties of the rectifying channel, there were considerable differences in the channel regulation data for cells derived from CF tissue. It is difficult to resolve the discrepancies between the conclusions of the two groups, especially since the same technique was used in both cases. These opposing results have stirred up great interest and neccessitate that the area of epithelial Cl transport regulatory mechanisms be scrutinized by other approaches.

Information on the regulation of the apical membrane Cl conductance also has been obtained using a varied repertoire of biochemical techniques. In the aforementioned study, Welsh and Liedtke demonstrated that, in CF derived cells, levels of cAMP before and after isoproterenol stimulation are similar to those found in normal cells under identical conditions. These data support the findings of Widdicombe (1986a) as well as the notion that the inability of CF tracheal epithelial cells to secrete Cl in response to β-adrenergic agonists is due to a defect distal to the point at which adenylate cyclase activity is increased and cAMP levels rise. To examine further the question of where the regulatory lesion in the pathway of channel activation would be, Barthelson and Widdicombe (1987) measured activity of protein kinase A in dog, cow, normal human, and CF tracheal epithelial cells grown in culture. These investigators found no differences between normal and CF derived cells, under baseline and maximally cAMP-stimulated conditions, in the activity of protein kinase A. Therefore, these results indicate that the Cl channel is regulated improperly at a site beyond

activation of cAMP-dependent kinase.

Schoumacher et al (1987) reached this same conclusion. They found that the catalytic subunit of protein kinase A, in the presence of ATP, when added to the solution bathing an excised inside-out patch of membrane from normal tracheal cells, induced activation of the rectifying Cl channel. The identical maneuver had no effect on patches from CF-derived cells. The presence of channels in these patches was verified independently by voltage-induced activation.[1] These studies thus favor a model in which the channel regulatory defect in CF tissue is beyond the point that kinase A activity is induced. Assuming the absence of any cytoplasmic factors in both normal and CF excised patches, unresponsiveness of CF Cl channels to exogenous kinase (in the presence of ATP) rests with a membrane associated defect. Shortly after this report, Li et al (1988) published similar findings.

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1. Schoumacher et al (1987) have noted that large depolarizing voltages are capable of evoking activity in silent channels in a patch. The method is now commonly used, at the end of a protocol, to determine if a patch has any channels.

Ion Dependences of the Basolateral Cl Uptake Pathway

Uptake of Cl via a mechanism coupled to the Na gradient has been of interest in many different cell types, including those of epithelial origin. Whether the Cl uptake pathway is K-dependent is a question which has remained unresolved for most of these cell types. Using isotope flux measurements, Russell (1983) has demonstrated that squid giant axon possesses a K-dependent NaCl cotransporter with the stoichiometry 2Na:1K:3Cl. This mechanism was subsequently demonstrated in ferret red blood cells (Hall and Ellory, 1985). Potassium dependence of NaCl cotransport has been documented in several epithelial tissues. An Na/K/2Cl cotransporter has been shown to be important in the proper salt secreting function of the elasmobranch rectal gland (Epstein and Silva,1985). These workers stress the enhanced energetic efficiency of such a mechanism over that of a NaCl cotransport system. However, several observations suggest that such a pathway is not the sole route for NaCl transport in other epithelial systems. Alvo et al (1985) demonstrated that K-independent NaCl cotransport exists, under isotonic conditions, in cells isolated from the medullary thick ascending limb of Henle's loop. This group subsequently presented evidence that imposition of hypertonic conditions on these cells resulted in uptake of Na and Cl which was dependent on the presence of K. In the intact perfused cortical thick ascending limb, it has been reported that luminal K was required for the active reabsorption of Na and Cl (Greger and Schlatter, 1981). In oxygen consumption studies of canine tracheal epithelium, Welsh (1984) compared the metabolic costs of Na and Cl transport; the data from these experiments support the presence of an Na/K/2Cl cotransport mechanism. Widdicombe et al (1983) demonstrated the presence of K-independent NaCl cotransport in dispersed cells from canine tracheal epithelium; this finding recently has

been confirmed for similarly prepared cells of bovine origin (Musch and Field, 1989). It is important to note that the dispersed cell preparation may induce artifacts in transport behavior related to the morphological changes associated with cell isolation. This mechanism apparently is important in the regulation of cell volume in response to osmotic shrinkage. Curiously, no increase in bumetanide-sensitive Na and Cl uptake was induced by the application of the secretagogue, epinephrine and the Ca^{++} ionophore, A23187. In contrast to these findings, Liedtke et al (1988) reported the presence of an α-adrenergic agent-and Ca^{++}-regulated, bumetanide-sensitive, basolateral Cl transporter in dispersed rabbit tracheal epithelial cells. These conflicting results suggest that the ion dependencies, and regulation of, basolateral membrane Cl transport are far from clearly resolved, and that epithelial cells possess substantial potential for heterogeneity of Na-coupled Cl transport pathways.

From observations such as these, it becomes apparent that K- dependence of Na gradient coupled Cl movement is a function of the particular stimulus for Cl transport. Clearly, there is a difference in the K-dependence of NaCl transport pathways involved in the processes of volume regulation and vectorial transport by epithelial cells. Thus, prior to a study of cotransporter ion dependence, it is necessary to define the transporter of interest in terms of its involvement in a specific process.

Purpose of the Present Study

The work documented in this dissertation focuses on the normal transport mechanisms responsible for the regulated secretion of Cl by the tracheal epithelium. This study was carried out under the premise that a thorough definition of the coordinated events underlying epithelial Cl secretion is necessary for understanding its malfunction in disease states. To this end, a variety of techniques have been employed where appropriate. A conscious attempt has been made to compare the conclusions derived from these studies with those made in work by others. The goal of this research has been to characterize and integrate further, on the level of the individual transport processes, the existing model of Cl secretion by tracheal epithelium.

Chapter Two

The Apical Membrane Cl Conductance

The conductive pathway for mucosal Cl exit from bovine tracheal epithelial cells was examined in purified apical membrane vesicles using the halide-sensitive fluorescent indicator, 6-methoxy- N-(3-sulfopropyl) quinolinium (SPQ).

Introduction

Apical and basolateral membrane vesicles have been useful systems for studying membrane transport, and have provided information complementary to that from studies in the intact epithelium (Hopfer, 1978; Murer and Hopfer, 1974; Wright, 1984). A method has been described for preparing apical membranes vesicles from the bovine tracheal epithelium (Langridge-Smith et al, 1983). The major transport processes of the intact tracheal apical membrane are a Cl conductance and an amiloride-sensitive Na conductance. Flux studies have shown both to be preserved in apical membrane vesicles (Langridge-Smith et al, 1983 and 1984). Recently, the Cl-sensitive fluorescent dye 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) has been used to study Cl transport in several vesicle systems (Chen et al, 1988 a and b; Illsley et al, 1988; Illsley and Verkman, 1987). The temporal resolution of this fluorescence assay (in the order of milliseconds and seconds in a stop-flow apparatus and a conventional fluorimeter, respectively) enables continuous monitoring of early phases of Cl influx in response to ion and pH gradients, as well as temperature changes and inhibitor addition. We report here the characterization of Cl transport in apical membrane vesicles isolated from bovine tracheal epithelial cells using the SPQ fluorescence quench technique.

Methods

Preparation of Apical Membrane Vesicles

Bovine tracheal epithelial cell apical membrane vesicles were prepared by a method modified from that described by Langridge-Smith et al (1983). For each preparation, 8-10 cow tracheae were removed immediately and put on ice at a local slaughterhouse. All subsequent procedures were carried out at 4°C. The tracheae were cut lengthwise through the cartilage, then rinsed in ice-cold solution A (table 1). The tracheae were then put into cold oxygenated solution A + 1 mM dithiothreitol (DTT) to remove adhering mucus from the epithelium. Prior to cell isolation, the tracheae were exposed to oxygenated solution A containing 2 mM EDTA. After 15 mins., the tissues were transferred to another beaker containing oxygenated solution A with 0.25 mM $MgCl_2$. Epithelial cells were isolated from the mucosa by scraping vigorously with either a siliconized glass microscope slide or a plastic scraper. The cells were collected in 100 mls of solution B (homogenizing buffer; table 1), pelleted at 300 x g for 8 min, and resuspended in Solution B. The suspension was homogenized with a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT) at 0.8 x top speed for 2 mins. Unbroken cells were pelleted in a 300 x g, 8 min spin and then re-homogenized. This mixture and the first supernatant were pooled, and constitute the initial homogenate against which all the subsequent partially purified fractions will be compared. Several differential centrifugation steps served to remove mitochondrial and nuclear membranes (Fig. 3). The crude microsomal pellet (figure 4a) was subjected to a Mg^{++} precipitation; this step further purified the membrane preparation by aggregating basolateral and other contaminating membranes which could then be spun down at low speed (figure 4b). Vesicles of a uniform size

Table 1

Solutions for Apical Membrane Vesicle Preparation

Solution	Composition	Additions
Solution A	150 mM NaCl 5 mM Tris-Sulfate pH 7.4	+/- 1 mM DTT +/- 2 mM EDTA +/- 0.25 mM $MgCl_2$
Solution B	100 mM Mannitol 2 mM Tris-Sulfate pH 7.5	+/- 10 mM $MgCl_2$ +/- 1 mM EDTA

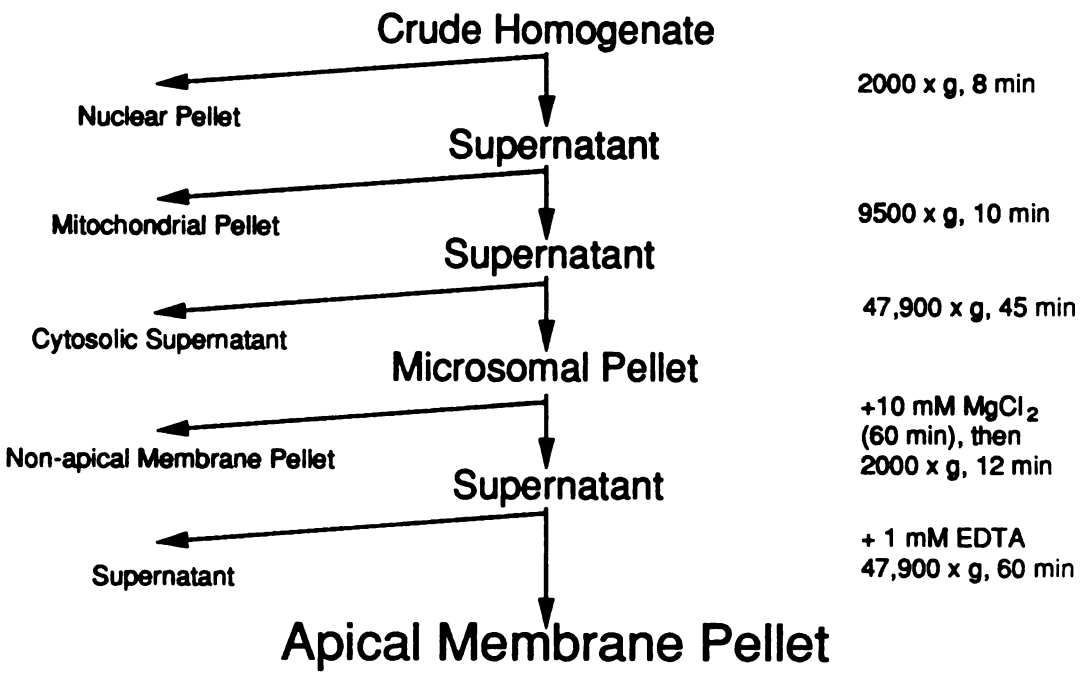

Figure 3. Schematic of bovine tracheal epithelial cell apical membrane vesicle preparation.

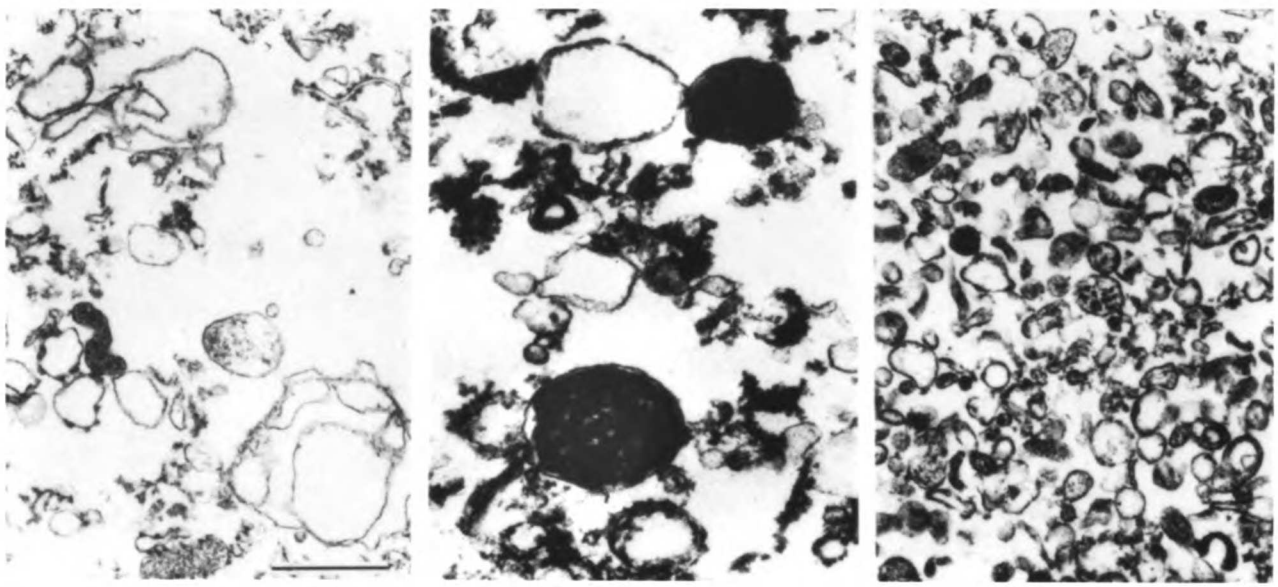

Figure 4. Transmission electron micrograph of membrane fractions. Left panel: crude microsomal pellet; middle panel: Mg^{++} precipitated membrane fraction; right panel: final apical membrane vesicles. Note uniformity in size and morphology. Scale bar equals 0.5 mm.

(0.2 μm diameter) and markedly increased homogeneity were obtained in the final pellet (figure 4c). The recovery of protein in the apical fraction, with respect to the levels in the initial homogenate, was ~ 1%, as determined using the protein assay of Smith et al (1985).

The purity of the apical membrane vesicles was assessed by comparing the activities of membrane enzyme markers with those of the crude initial homogenate and fractions of membranes removed during the preparation. Apical membranes are identified by an accumulation of alkaline phosphatase. Na/K ATPase activity is indicative of basolateral membranes while succinic dehydrogenase and DNA are associated with mitochondrial and nuclear membranes, respectively.

Assay of alkaline phosphatase activity is based on the following reaction:

$$\text{nitrophenylphosphate} + H_2O \longrightarrow \text{p-nitrophenol} + H_3PO_4.$$

This dephosphorylation is catalyzed, at pH 10.5, by the action of alkaline phosphatase. As the reaction progresses, p-nitrophenylphosphate, a colorless compound, becomes dephosphorylated to yield p-nitrophenol, which is yellow. The colorimetric change is monitored at 405 nm (Bessey, O.A. et al, 1946). Na/K ATPase levels can be assessed using an ATP regenerating assay based on that of Schoner et al (1967). Briefly, in the presence of Mg, Na, and NH_4, ATPase activity produces ADP and P_i from ATP. The regenerating system contains phosphoenolpyruvate and pyruvate kinase; in the presence of ADP, these permit the recycling of ADP to ATP, as well as formation of pyruvate. Lactate dehydrogenase acts as the catalyst for the oxidation of NADH to NAD, with the concomitant production of lactate from pyruvate. The decrease in NADH levels is

associated with a change in optical density at 340 nm, and is directly related to the ATPase activity. The ouabain-sensitive fraction of the total ATPase activity represents that attributable to Na/K ATPase. Succinic dehydrogenase activity can be determined by measuring the malonate inhibitable change in optical density at 600 nm observed by addition of succinate and KCN to the sample (Ackrell et al, 1978). The fluorescence of 33258 Hoechst is increased greatly upon complexation with DNA. This property forms the basis of a sensitive quantitative assay for DNA (Cesarone et al, 1979). In this procedure, excitation and emission wavelengths are 360 nm and 450 nm, respectively. The data are summarized in table 2 and indicate that the final membrane fraction is constituted primarily of apical membranes.

That the apical membrane preparation consisted of vesicles was revealed by demonstration of an osmotically active space. Vesicles (98 μg protein) were preincubated with 0.48 μCi $Na^{36}Cl$ in buffers (200 μl) with varying osmotic strengths. Buffers consisted of 1000, 500, 200, 100, 67, or 50 mM mannitol, with 2 mM Tris-sulfate, pH 7.5. After a 1 hr. equilibration period at room temperature, 200 μl of reaction mixture was removed and applied on a prewetted 0.22 μm pore size Nucleopore filter (Pleasanton, CA) fitted on a filtration manifold (Millipore, Bedford, MA). The sample was washed rapidly with 2 mls of ice-cold 100 mM Mannitol, 2 mM Tris-sulfate, pH 7.5, with DPAC (10^{-3} M) added. As controls, the following conditions were also tested. A mixture of vesicles in 50 mOsm buffer and 0.1% Triton X-100 was subjected to treatment identical to other samples. Counts associated with this sample would represent non-specific binding to lysed membranes. A sample to which vesicles were added immediately prior to filtration (i.e. no preincubation) established a 0-time point. A reaction mixture to which no vesicles were added prior to filtration provided information on the extent of non-selective tracer binding to the filters. After removal of filters from

Table 2

Enzyme Activities

Enzyme	Accumulation Ratio (± SEM)
DNA	0.3 ± 0.3 (n=4)
SDH	0.7 ± 0.4 (n=4)
Na/K ATPase	3.4 ± 1.7 (n=2)
Alkaline Phosphatase	11.4 ± 1.8 (n=6)

the manifold and transferral to vials, Safety-Solve scintillation fluid (R.P.I., Mount Prospect, Il) was added to the samples. The filters were counted for ^{36}Cl in a Beckman scintillation counter (model LS7500; Beckman Instruments, Irvine, CA). Counts per minute were plotted as a function of the reciprocal of buffer osmolarity. The results (Fig. 5) indicate the presence of sealed vesicles; a small osmotically active space in hypertonic solutions increased as a function of lowering buffer tonicity. Tracer levels were dramatically lowered in the presence of Triton X-100, indicating that the increase in counts in hypotonic medium indeed was due to an increase in vesicle volume, and not in nonselective associations.

The vesicles were suspended in 100 mM mannitol, 2 mM Tris-Sulfate, pH 7.5, aliquoted, and stored at -80°C until use. Storage at -80°C had no effect on transport characteristics.

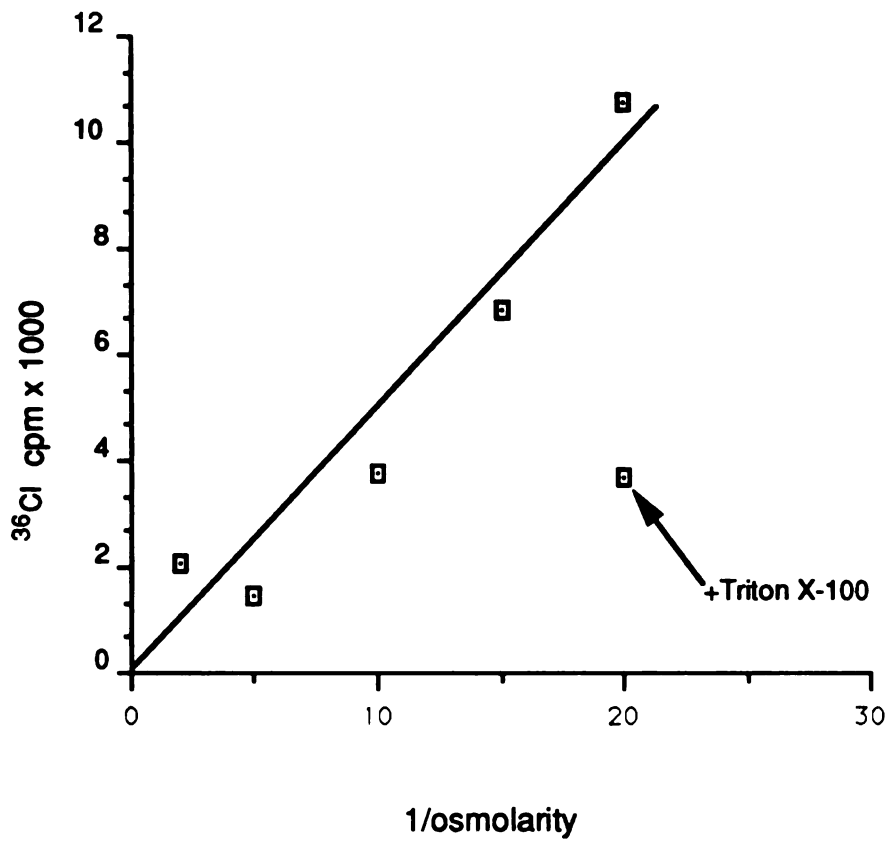

Figure 5. Membrane Vesicles prepared in the method of Langridge-Smith et al (1983) demonstrate the presence of an osmotically active space. Conditions of experiment given in text.

Measurement of Cl Influx

Vesicular Cl influx was measured using the SPQ fluorescence quench method previously described by Illsley and Verkman (1987). The fluorescence of SPQ is decreased in the presence of halide anions. The rapid detection of small changes in Cl levels afforded by this technique enabled characterization of apical Cl transport in much greater detail than previously possible with tracer flux measurements.

SPQ was synthesized as described previously (Wolfbeis and Urbano, 1982). Apical membrane vesicles were loaded with SPQ and an appropriate 0-Cl buffer by incubation for >24 hr. at 4°C. Loading buffers contained 100 mM sucrose, 10 mM Tris-Hepes, X mM K-Gluconate, and (150-x) mM N-Methyl-D-Glucamine Gluconate (NMG-Gluconate), pH 7.0. X= 50 mM in experiments where membrane potential (ψ) was clamped at 0 mV; x= 5mM when ψ was set to 60 mV. Immediately before the experiment, the vesicles were washed with three consecutive cycles of ultracentrifugation (60,000 g x 10 mins., 4°C) and resuspension in SPQ-free 0-Cl buffer to remove external SPQ. The final pellet was put up in a small volume of 0-Cl buffer to bring the protein concentration to 8 mg/ml. The vesicle suspension was kept on ice until the time of the experiment to minimize dye leakage (<5% per hour). Vesicles were used within 4 hrs. Valinomycin (25 µg/mg protein) was added as an ethanolic stock to the vesicles immediately after final resuspension to clamp the vesicles at the K equilibrium potential. Thus, by adjusting the amount of K in the preincubation and transport buffers, the vesicular membrane potential (V_m) could be clamped at any desired voltage.

External buffers had the following composition: 100 mM sucrose, 10 mM Hepes-Tris, and a total of 150 mM salt, 50 mM of which was always K-Anion. The remaining 100 mM salt was NMG-Gluconate except in studies testing the

effect of cis Na, when 50 mM Na-Gluconate and 50 mM NMG-Gluconate were used. For all experiments pH was 7.0, except those in which pH gradients were employed. Gluconic acid (1 mM) was used to titrate to pH 5.5. Internal and external buffers were balanced for both osmolarity and ionic strength. All buffers were filtered twice with 0.22 μM Millipore (Bedford, MA) or Nucleopore (Pleasanton, CA) filter discs to remove dust particles which scatter light.

Chloride transport blockers and their sources were: H_2DIDS (dihydro-4,4'-diisothiocyano-2,2'-disulfonic stilbene), Molecular Probes (Eugene, OR); furosemide, Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ); and DPAC (diphenylamine-2-carboxylate), ICN Biomedicals (Plainview, NY). Vesicles were preincubated with H_2DIDS or furosemide (both 0.1 mM) for at least an hour. DPAC was added directly from a 100 mM ethanolic stock to a final concentration of 0.2 mM.[2] Addition of an equal volume (4 μl) of ethanol had no effect.

SPQ fluorescence was measured continuously and averaged at 1 sec intervals with an SLM 4800 fluorimeter (SLM, Urbana, IL) interfaced to an IBM PC/XT computer. Acrylic cuvettes (Sarstedt, FRG) were used in all experiments. Excitation wavelength was 350 nm (8 nm bandpass); emission fluorescence was measured with a Schott KV 408 cut on filter (Duryea, PA). Two ml of uptake buffer was added to a cuvette positioned in a thermostatically controlled holder and stirred continuously. To initiate the experiments, apical membrane vesicles (~40 μg protein) were added rapidly to the cuvette. Fluorescence was monitored

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2. Higher concentrations of DPAC were not used because of its high extinction coefficient (~ 6900 M^{-1} cm^{-1}) at 350 nm. At the concentration used, 0.2 mM, effects due to absorbance on the fluorescence measurements were negligible.

for 800 sec., at which time the fluorescence signal remained constant and was regarded as representative of complete equilibration of external Cl with the intravesicular space. In some experiments, complete equilibration was achieved by the addition of Triton X-100 (Fig. 6). There was no further decrease upon Triton addition after 800 sec.

Chloride influx, d[Cl]/dt (mM/sec), was calculated from the initial rate of change of SPQ fluorescence and a two-point calibration method using an SPQ fluorescence versus [Cl] curve as described previously (Illsley and Verkman, 1987). Briefly, the fluorescence of SPQ can be related to the concentration of Cl by the Stern-Volmer equation:

$$F = F_{ex} + F_o/(1 + K[Cl]) \qquad (1)$$

where K is the Stern-Volmer quench constant, F_{ex} is the time-independent fluorescence of extravesicular SPQ, F is the fluorescence of intravesicular SPQ in the presence of Cl, and F_o is the fluorescence of intravesicular SPQ in the absence of Cl. This equation can be differentiated and rearranged to yield:

$$(d[Cl]/dt)_{t=0} = F_o/[K(F - F_{ex})^2](dF/dt)_{t=0} \quad (2)$$

which reduces to:

$$(d[Cl]/dt)_{t=0} = (1/KF_o)(dF/dt)_{t=0} \qquad (3)$$

Knowing the initial rate of change of SPQ fluorescence ($(dF/dt)_{t=0}$), F_o, F_{ex}, and F, d[Cl]/dt (mM/sec) at t = 0 can be determined. F_o and F_{ex} are determined as illustrated in Fig. 6. J_{Cl} in nmol.sec^{-1}.mg protein^{-1} was calculated by multiplying d[Cl]/dt (mM/sec) by the glucose space of the vesicles, (2.4 µl/mg protein; unpublished data). J_{Br} and J_I were calculated analogously (Illsley and Verkman, 1987).

Calculation of Cl Flux

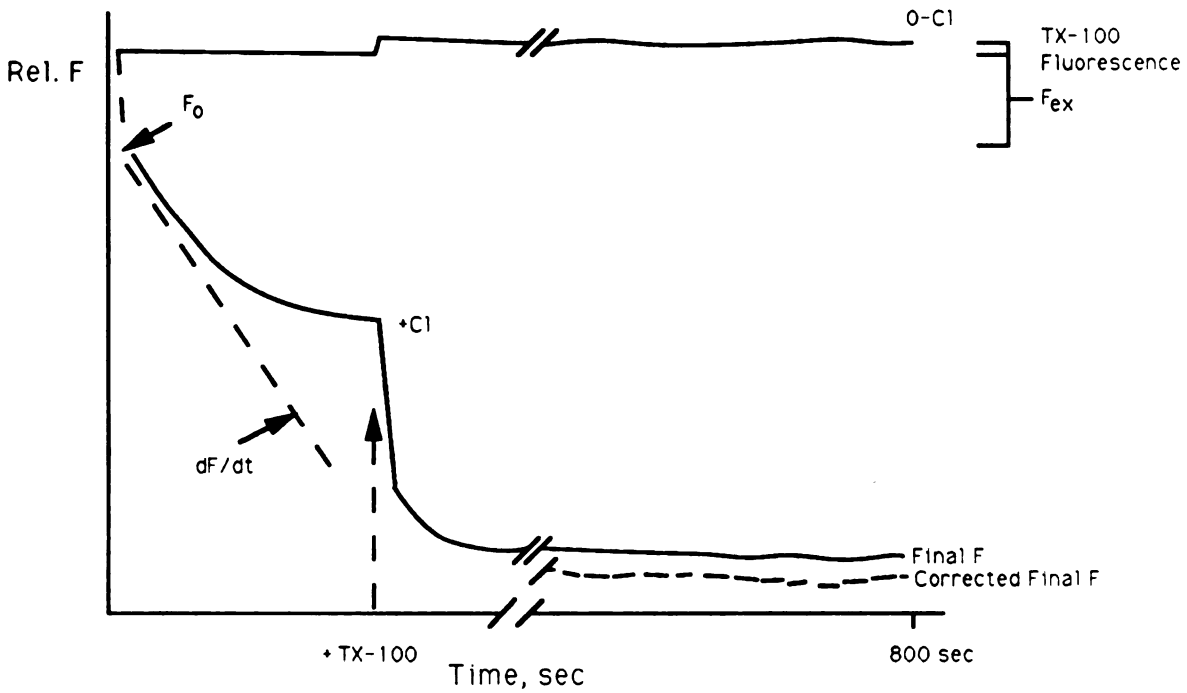

Figure 6. Parameters for the calculation of Cl flux are illustrated in this composite figure. Final fluorescence is determined by following fluorescence until t=800 sec or by lysing the vesicles with Triton X-100 to equilibrate intravesicular SPQ with the external buffer. Note that Triton X-100 has a small degree of associated fluorescence; this is subtracted from final values obtained via vesicle lysis with this detergent.

The Stern-Volmer quench coefficient used to calculate d[Cl]/dt in the presence of gluconate has been determined previously (Illsley and Verkman, 1987). SPQ fluorescence is mildly quenched by gluconate. A change in gluconate concentration will change the Stern-Volmer quench constant, K, in a manner described by the relationship:

$$K_{[gluc]} = K_{[gluc]=0}/(1.0+ 0.007[gluc]) \quad (4)$$

where the gluconate concentration, [gluc], is in mM. $K_{[gluc]=0}$, the quench constant in the absence of gluconate, is 0.118, 0.197, and 0.282 for Cl, Br, and I, respectively. For an internal gluconate of 150 mM, Stern-Volmer quench constants of 0.058, 0.097, and 0.138 mM^{-1}, respectively, were used for calculations of J_{Cl}, J_{Br}, and J_I. (Illsley and Verkman, 1987). Statistical significance was determined with Student's t-test. P of < 0.05 was considered statistically significant. Data are expressed as mean ± SEM.

Results

Fig. 7 shows the time course of fluorescence quenching on addition of SPQ-loaded vesicles to a medium containing 50 mM Cl. The initial rate of quenching was increased in the presence of a +60 mV membrane potential at 23°C. In seven experiments on two sets of vesicles, a 60 mV inside positive membrane potential increased J_{Cl} significantly from a control value of 0.32 ± 0.12 nmol.sec^{-1}.mg protein^{-1} (when $\psi = 0$ mV) to 0.50 ± 0.07 nmol.sec^{-1}.mg protein^{-1} (Fig. 8).

Diphenylamine-2-carboxylate (DPAC) has been shown to inhibit Cl transport in a variety of epithelia (DiStefano et al, 1985). In cultured canine tracheal epithelial cells, DPAC inhibits single channel current and short circuit current (I_{sc}) with a K_d of about 1 mM (Welsh, 1986b). We have shown DPAC to inhibit I_{sc} in monolayers cultured from bovine tracheal epithelial cells with a similar K_d (data not shown). As shown in figure 8, DPAC (0.2 mM) significantly inhibited J_{Cl} by $34 \pm 7\%$ (n = 5), compared to an inhibition of about 20% predicted from the K_d. These data support the presence of a DPAC-sensitive Cl conductance similar to that demonstrated in the apical membranes of the intact tracheal epithelial cells.

Other possible pathways for Cl influx were examined. To determine whether Na/K/2Cl cotransport was present, Na was added to the external buffer. There was no significant enhancement of J_{Cl} by cis-Na (mean per cent change = -0.9 ± 4.9 %; n = 6; Fig. 8) over the control rate of 0.38 ± 0.03 nmol.sec^{-1}.mg protein^{-1}. Furthermore, the loop diuretic furosemide (0.1 mM) had no effect on J_{Cl} in both the presence and absence of external Na. These results indicate the absence of Na/K/2Cl and Na/Cl cotransport in apical membrane vesicles from bovine trachea. Cl/OH(H) transport has been shown to be an important mechanism of Cl transport in several systems. However, an inwardly directed proton

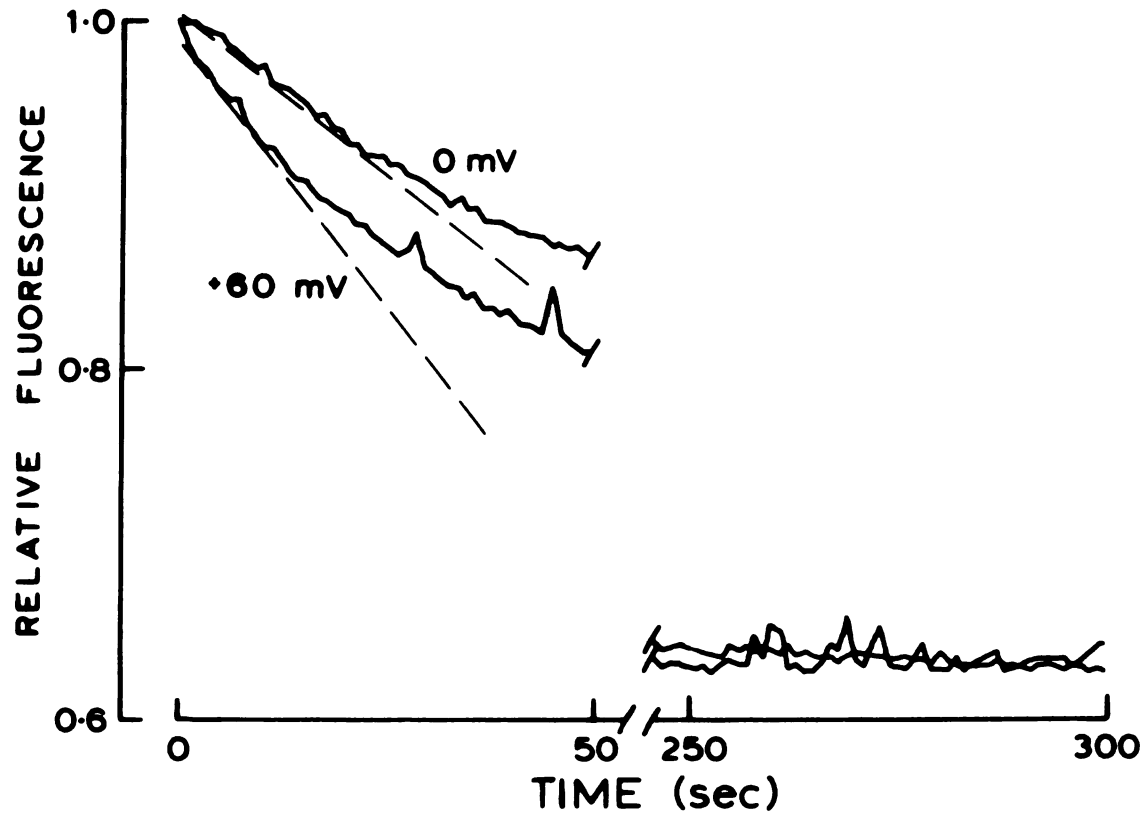

Figure 7. Effect of changing membrane potential on the time course of intravesicular SPQ fluorescence in response to a 50 mM Cl gradient. Upper curve is time course under control conditions (0 mV membrane potential; intravesicular and external [K] = 50 mM). Lower curve is time course in the presence of a +60 mV membrane potential (intravesicular [K] = 5 mM; external K] = 50 mM). In these runs, Triton X-100 was added at 150 sec to obtain final fluorescence of SPQ. Initial rates of fluorescence decrease (dotted lines) were determined by using a computerized exponential fit to the first 90 points. Records are paired runs from the same vesicle preparation.

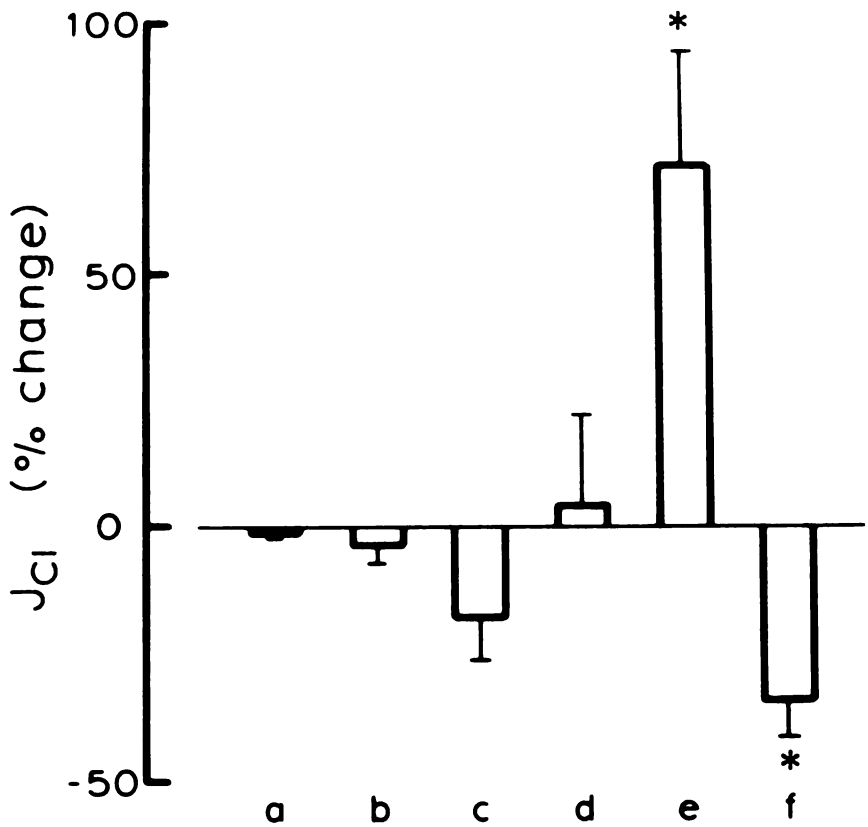

Figure 8. Effects of a) 50 mM cis Na, b) 0.1 mM furosemide, c) 0.1 mM H_2DIDS, d) an inwardly directed proton gradient of 1.5 pH units and 0.1 mM H_2DIDS, e) a $^+$60 mV membrane potential, and f) 200 mM DPAC on J_{Cl}. Experiments were performed at 0 mV internal potential difference and 37°C, except that experiments comparing 0 and $^+$60 mV were done at 23°C. Data are presented as the mean percent change from control J_{Cl} (± SEM). Asterisks denote significance ($p < 0.05$).

gradient (pH_{ext} = 5.5, pH_{int} = 7.0) had no effect on J_{Cl} (test J_{Cl} = 0.31 ±0.02 nmol.sec^{-1}.mg protein^{-1}; control J_{Cl} = 0.29 ± 0.02 nmol.sec^{-1}.mg protein^{-1}; n = 2). Preincubation with the stilbene anion exchange inhibitor, H_2DIDS (0.1 mM), did not alter J_{Cl} significantly (-18 ± 9% from control; n = 4). Finally, a pH gradient and H_2DIDS in combination did not change J_{Cl}. These results indicate absence of Cl/OH(H) transport in our membrane preparation.

To characterize further the basic properties of the Cl conductance, the effects of external Cl concentration and temperature on J_{Cl} were examined. The results (Fig. 9a) show saturation of J_{Cl} with increasing external Cl. The apparent K_d was 24 mM with a V_{max} of 0.54 nmol.sec^{-1}.mg protein^{-1}. Figure 9b shows the temperature dependence of J_{Cl} in the form of an Arrhenius plot. The plot yields an activation energy (E_a) for Cl influx of ~5 kcal/mol.

Initial rates of anion transport in response to 50 mM gradients of Cl, Br, and I were compared. J_{Br} was similar to J_{Cl} (0.37 ± 0.02 nmol.sec^{-1}.mg protein^{-1} vs. 0.48 ± 0.03 nmol.sec^{-1}.mg protein^{-1}, n = 7). There was no difference between J_I and J_{Cl} (0.41 ± 0.11 nmol. sec^{-1}.mg protein^{-1} and 0.47 ± 0.05 nmol.sec^{-1}.mg protein^{-1}, respectively; n = 6). In one set of vesicles, halide fluxes were determined in the presence of a +60 mV internal potential difference. Again, no difference in fluxes of the three halides was detected (data not shown).

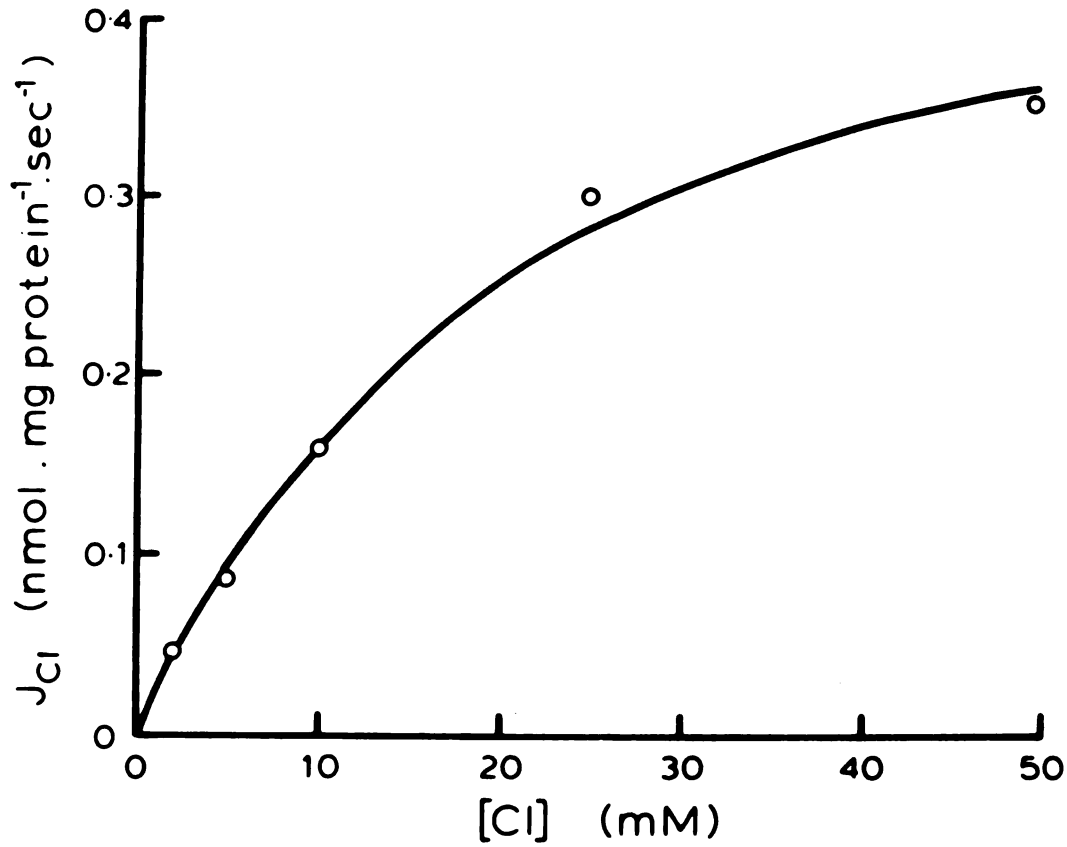

Figure 9a. Dependence of J_{Cl} on Cl concentration. Curve is the best least squares fit to an Eadie-Hofstee plot of data, with a K_d = 24 mM and V_{max} = 0.54 nmol.sec^{-1}.mg protein^{-1}. Points are means of triplicate estimates; standard errors were approximately the same size as the symbols used.

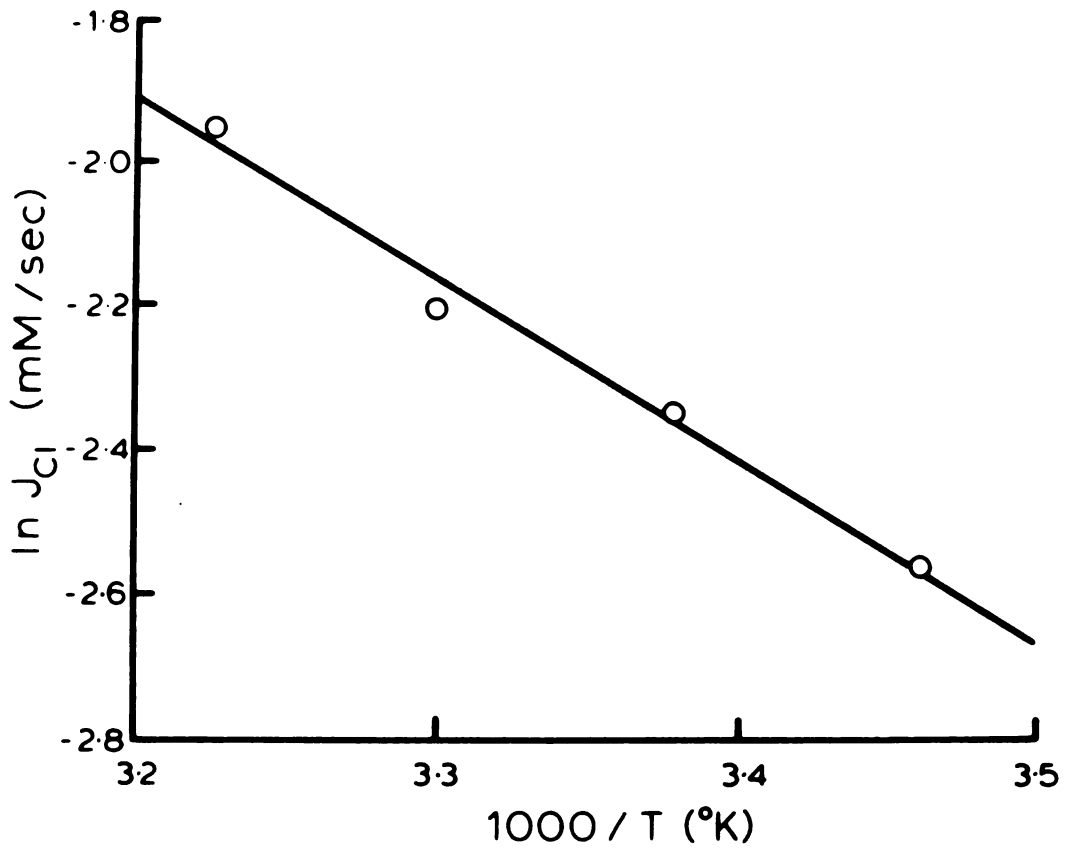

Figure 9b. Temperature dependence of J_{Cl}. The calculated E_a was 5.0 ± 1.3 kcal/mole. Data points are the means of triplicate estimates. Similar results were obtained in three other sets of studies.

Discussion

We report here the characterization of the transport properties of apical membranes prepared from bovine tracheal epithelial cells using a fluorescence assay. Other studies utilizing this fluorescence technique in renal brush border (Chen et al, 1988a) and basolateral (Chen et al, 1988b) membranes, as well as red blood cell ghosts (Illsley and Verkman, 1987) and placental brush border membranes (Illsley et al, 1988) have established the accuracy of Cl flux measurements using SPQ. Compared to isotopic methods, the SPQ fluorescence assay affords greater sensitivity and temporal resolution, while requiring less membrane protein.

Compared to carriers or pumps, ion channels have very high turnover numbers and correspondingly low membrane densities. This creates two potential problems in studying their properties using membrane vesicles. Firstly, because of their small size, many vesicles may lack channels. Secondly, in those vesicles which do contain channels, the equilibration of tracer in such a small volume may be too rapid, and prove difficult to detect. Simple calculations suggest that these concerns are unimportant in the experiments described here.

Tracheal epithelium treated with indomethacin has an apical membrane conductance (G_a) of 0.44 $mS.cm^{-2}$, as revealed by equivalent circuit analysis (Welsh et al, 1983). Under these conditions there is no net Cl secretion and I_{sc} is equal to net Na absorption. When maximal Cl secretion is induced by epinephrine, G_a increases to 3.64 $mS.cm^{-2}$, though Na absorption remains unchanged. This change in conductance is not seen in Cl-free medium. Thus the additional 3.24 $mS.cm^{-2}$ probably represents opening of Cl channels. Apical membrane Cl channels have unitary conductances of 20-50 pS (Frizzell et al, 1986a; Welsh, 1986a and b). In tissues stimulated with cAMP, the probability of opening (P_o)

for Cl channels is about 0.15.[3] Our vesicles have a diameter of approximately 0.2 μm, corresponding to a surface area of 0.13 μm^2. These figures yield an average of one Cl channel per vesicle.

The half time for influx can be estimated from:

$$t_{1/2} = (U_{max} \cdot \ln 2)/(n \cdot P_o \cdot J) \quad (5)$$

where J is the influx in ions . sec^{-1} for an open channel, U_{max} is the maximal Cl uptake (ions), n is the number of channels per vesicle, and P_o is the probability of opening.

The diffusional flux in the absence of a membrane potential in $\mu Eq.hr^{-1}$ is within 1% of the conductance in mS at 37°C (Linderholm, 1952; Schultz et al, 1964). Thus, a 30 pS channel corresponds to an influx (J) of 5×10^6 ions per second. Assuming 50 mM internal Cl and a single 30 pS channel per vesicle, figure 10 shows how the $t_{1/2}$ depends on vesicle size and the P_o for the channel. Vesicles used in the present study have a diameter of 0.2 μm. Therefore, 50 mM Cl corresponds to 120,000 ions ($=U_{max}$). Substitution of this value, together with J = 5×10^6 ions/sec, n = 1, and P_o = 0.15, into equation 5 gives a $t_{1/2}$ of 111 msec for the cAMP stimulated state.

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3. In T_{84} cells stimulated to secrete by raising intracellular cAMP, P_o has been estimated as 0.15 (Frizzell et al, 1986a). Welsh (1986b) found Cl channels in 43 excised, inside-out patches of apical membrane from cultured dog tracheal epithelial cells. In the cell-attached mode, only 8 of these patches showed Cl channels following addition of isoproterenol. Thus, even in the stimulated state, the overall P_o for this population of channels is < 0.2. Similar results have been obtained for human tracheal cells in culture; only a small fraction of Cl channels open in the cell-attached mode in response to isoproterenol, and once open, the P_o is ~0.5 (Welsh, 1986a). From these figures, and similar results of Frizzell et al (1986b), P_o after elevation of cAMP can be estimated to be ~0.15.

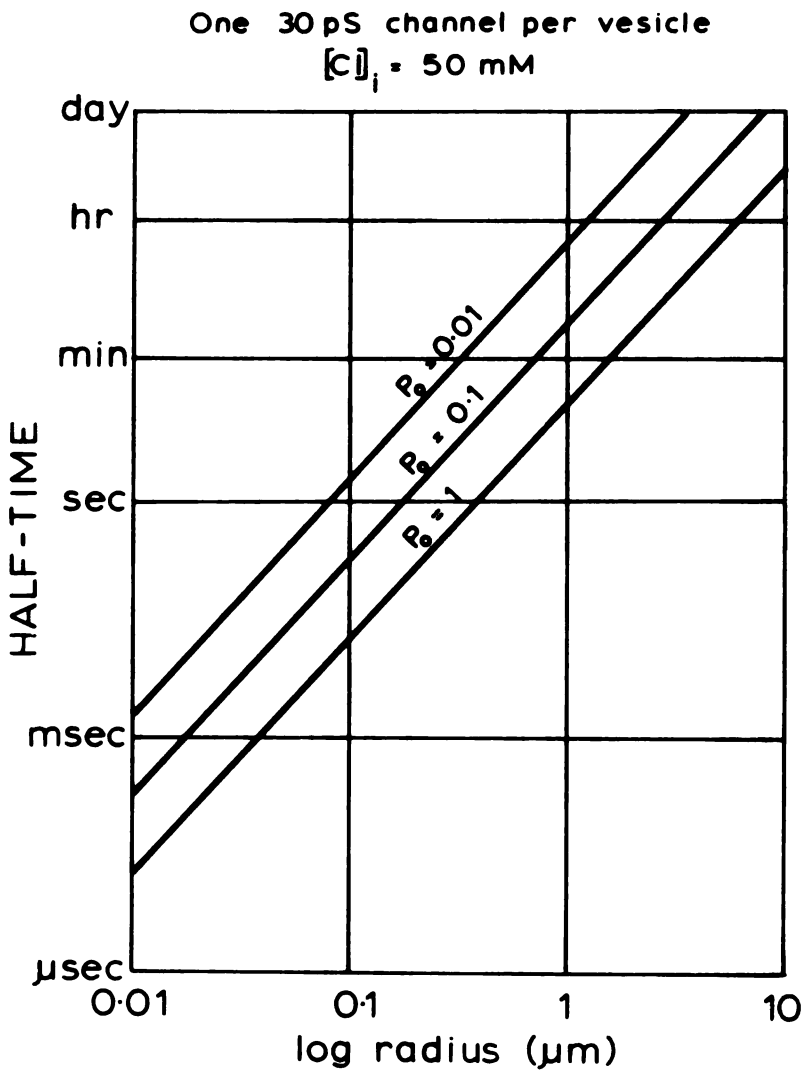

Figure 10. Plot of $t_{1/2}$ as a function of P_o and vesicle size, assuming one 30 pS channel per vesicle.

Our experiments, as well as the Cl efflux studies of Dubinsky and Monti (1986), yield a value for $t_{1/2}$ of ~ 10 sec. Therefore, the resting P_o is about one hundredth of the value of P_o in the stimulated state, or ~ 0.0015. This low value for P_o in the absence of cAMP-dependent activation is consistent with both equivalent circuit and patch clamp data. In an equivalent circuit analysis of dog tracheal epithelium, Welsh et al (1983) found that elevation of cAMP levels with epinephrine produced an apical membrane electromotive force (E_a) that was essentially equal to the equilibrium potential for Cl across this membrane. With cAMP levels lowered by prolonged treatment with indomethacin, the apical membrane resistance increased ten-fold. This membrane now behaved as if perfectly Na-selective, with E_a being approximately the same as E_{Na}, the equilibrium potential for Na (Welsh et al, 1983).[4] In cell-attached patches, prior to addition of isoproterenol, all (Frizzell et al, 1986b) or almost all (Welsh, 1986a and b) Cl channels from either human or dog showed no openings.

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4. An estimate for the resting G_{Na} of the apical membrane can be determined from the relation:

$$I_{Na} = G_{Na} (\psi_a - E_{Na}) \quad (6)$$

where I_{Na} is the I_{sc} in the resting state (which is due entirely to Na transport). Using values for I_{Na} of 30 - 60 $\mu A.cm^{-2}$, ψ_a of -50 mV, and E_{Na} of +50 mV, one obtains estimates for G_{Na} of 0.3 - 0.6 $mS.cm^{-2}$. This is similar to the estimate of G_a in resting tissues of ~0.4 $mS.cm^{-2}$ obtained by Welsh et al (1983), again suggesting that most of the resting G_a is due to Na.

The Cl fluxes measured in this study permit the calculation of the absolute Cl permeability (P_{Cl}). Using the glucose space of 2.4 μl.mg protein^{-1} and the surface area-to-volume ratio for a 0.2 μm diameter sphere of 3 X 10^5 cm^{-1}, the control J_{Cl} of ~0.35 nmol.sec^{-1}.mg protein^{-1} (37°C) is equivalent to a transmembrane flux of 0.49 pmol.cm^{-2}.sec^{-1}. With a membrane potential of 0 mV and a [Cl] of 50 mM, this corresponds to a P_{Cl} of 9.8 X 10^{-9} cm.sec^{-1}. The conditions of the experiments of Langridge-Smith et al (1984) do not permit the rigorous calculation of P_{Cl} because valinomycin (5 μg/ml) was present in the absence of intravesicular K. Thus, the electrochemical driving force is not defined. However, using ^{36}Cl, these investigators obtained an initial rate of Cl flux of 0.18 nmol.sec^{-1}.mg protein^{-1} with a 20 mM Cl gradient. Once this value is normalized to the 50 mM Cl gradient used in our experiments, this is in close agreement with our data. Dubinsky and Monti (1986), using a Cl-sensitive electrode to measure Cl efflux from vesicles loaded with 150 mM Cl, identified a valinomycin-stimulated efflux of 4 nmol.mg protein^{-1}. sec^{-1} as being via a conductive pathway. However, in these experiments valinomycin (1 μg/ml) was present and the vesicles contained 160 mM K with no external K. The vesicles must therefore have possessed a considerable interior negative membrane potential, which would drive Cl efflux.

Our results exclude the presence of Na/K/2Cl (or Na/Cl) cotransport in tracheal apical membrane vesicles, as indicated by the lack of effect on J_{Cl} by cis-Na and/or 0.1 mM furosemide. At first, this finding is somewhat surprising in light of the slight enrichment of basolateral membranes in the vesicle preparation, indicated by an accumulation ratio of 3.4 for the Na/K ATPase. However, basolateral vesicles are much larger than their apical counterparts; therefore, equilibration of 50 mM Cl in these membranes is much slower. Coupled to the far greater enrichment of the apical membrane marker, alkaline phosphatase

(11.4), it follows that initial d[Cl]/dt is primarily due to Cl transport via a channel. Thus, either inhibition (by furosemide) or stimulation (by cis-Na) of the basolaterally derived, slow component to influx will have very minor effects on the measured initial rate of fluorescence quench, dF/dt. Taken together, these findings support the existing model for tracheal epithelial cell Cl secretion (Welsh, 1987), which localizes Na/K/2Cl (or NaCl) cotransport to the basolateral, rather than the apical membrane of the cell (Widdicombe et al, 1983).

In our preparation, Cl/OH(H) transport does not contribute significantly to J_{Cl}; an inwardly directed proton gradient, 0.1 mM H_2DIDS, and a combination of the two did not change J_{Cl} significantly. Our findings are consistent with observations in cultured bovine tracheal epithelia, where H_2DIDS had no effect on I_{sc} (data not shown). Neither SITS nor DIDS affects ^{36}Cl uptake into apical membrane vesicles (Langridge-Smith et al, 1984).

The data obtained in this study support the presence of a voltage-driven Cl conductance; K-valinomycin voltage clamping of the membranes to a potential of +60 mV resulted in a significant increase in J_{Cl}. In cultured (data not shown) and intact (Langridge-Smith, 1986) bovine tracheal epithelium, addition of DPAC or related compounds to the mucosal side of the tissue decreased Cl transport. DPAC also has been reported to attenuate the current amplitude of Cl channels in excised, inside-out patches from cultured canine epithelial cells (Welsh, 1986b). In our studies, 0.2 mM DPAC blocked J_{Cl} by approximately 34%, providing further support for conductive Cl entry.

The activation energy for Cl transport, 5.0 kcal/mole, is comparable to that for free diffusion of Cl in water but is much less than that for a carrier (~20-30 kcal/mol) (Parsegian, 1969; Armstrong, 1975), supporting transport via a pore, rather than a carrier. ^{36}Cl fluxes into apical membrane vesicles are temperature-dependent (Langridge-Smith et al, 1984), however, difficulties in determining

accurate initial rates of tracer uptake did not permit calculation of the activation energy.

Values for J_{halide} as calculated from SPQ fluorescence were similar for Cl, Br, and I. Recently, Frizzell (1987) has reported that the two different apical membrane Cl channels of tracheal epithelium (50 pS and 20 pS) have differing selectivity: I (1.6), Br (1.3), Cl (1) and Cl (1), Br (0.4), I (0.4), respectively. Our results (Cl ~ I ~ Br) may reflect an average sequence from these two channel types.

In conclusion, Cl transport in vesicles derived from the apical membranes of bovine tracheal epithelial cells proceeds via a pathway that is conductive and DPAC-sensitive. No significant contribution by electroneutral Na/K/2Cl cotransport and Cl/OH(H) transport was observed. The dependence of initial flux rates on Cl concentration shows saturation. The calculated E_a supports the existence of a pore, rather than a carrier-type conductive mechanism. The influx pathway has similar selectivity for Cl, Br, and I. Finally, these results provide further support for the use of vesicles from bovine tracheal epithelium in studies of the apical membrane chloride channel and its regulation.

Summary

Apical membranes were prepared by a modification of the technique of Langridge-Smith et al (1983). With an inwardly directed 50 mM Cl gradient at 23°C, the initial rate of Cl entry (J_{Cl}) was increased significantly from 0.32 ± 0.12 nmol.sec^{-1}.mg protein^{-1} (mean ± SEM) to 0.50 ± 0.07 nmol.sec^{-1} mg protein^{-1} when membrane potential was changed from 0 to +60 mV with K/valinomycin. At 37°C, with membrane potential clamped at 0 mV, there was a 34 ± 7% (n = 5) decrease in J_{Cl} from a control value of 0.37 ± 0.03 nmol.sec^{-1}.mg protein^{-1} upon addition of 0.2 mM diphenylamine-2-carboxylate. The following did not alter J_{Cl} significantly (J_{Cl} values given as per cent change from control): 50 mM cis Na (-1 ± 5%), 0.1 mM furosemide (-3 ± 4%), 0.1 mM furosemide in the presence of 50 mM cis Na (-5 ± 2%), 0.1 mM H_2DIDS (-18 ±9%), a 1.5 pH unit inwardly directed H gradient (-7 ± 7%), and 0.1 mM H_2DIDS in the presence of a 1.5 unit pH gradient (4 ± 18%). With inward 50 mM anion gradients, the initial rates of Br and I entry (J_{Br} and J_I respectively) were not significantly different from J_{Cl}. J_{Cl} was a saturable function of Cl concentration with apparent K_d of 24 mM and apparent V_{max} of 0.54 nmol.sec^{-1}.mg protein^{-1}. Measurement of the temperature dependence of J_{Cl} yielded an activation energy of 5.0 kcal/mol (16-37°C). These results demonstrate that Cl transport in tracheal apical membrane vesicles is voltage-dependent and inhibited by diphenylamine-2-carboxylate. There is no significant contribution from the Na/K/2Cl, Na/Cl, or Cl/OH(H) transporters. The conductive pathway does not discriminate between Cl, Br, and I and is saturable. The low activation energy supports a pore-type mechanism for the conductance.

Chapter Three

Regulation of Apical Membrane Cl Conductance

The question of how the apical Cl channel is regulated was next examined in bovine tracheal epithelial cell apical membrane vesicles using a combination of the SPQ fluorescence quench assay and the hypotonic lysis method for macromolecular entrapment developed by Illsley et al (1988). Specifically, the roles of two possible mediators were investigated—the catalytic subunit of protein kinase A and the GTP-activated G proteins.

Introduction

Regulation of the conductive Cl transport pathway in apical membranes of tracheal epithelial cells recently has become an area of interest, largely due to studies demonstrating its improper functioning in the genetic disease, cystic fibrosis (CF) (Barthelson and Widdicombe, 1987; Widdicombe, 1986a; Welsh, 1986a; Welsh and Liedtke, 1986; Frizzell et al, 1986b; Frizzell, 1987). Many of these studies were done using single channel methods on excised patches of membrane from cultured tracheal cells of both normal and CF origin. Chloride channels in patches excised from normal human tracheal epithelial cells are opened by bath application of the catalytic subunit of protein kinase A, in the presence of ATP. This effect is not observed in CF cells. There are, however, conflicting data concerning the role of intracellular calcium in the regulation of this channel.

G proteins have been demonstrated, in a variety of cell types, to activate ion channels directly. Recently, in this laboratory, Yang has shown that bovine tracheal epithelial apical membranes prepared by the method of Langridge-Smith et al (1983) possess a protein component that is ADP ribosylated by pertussis toxin (see Fig.11) and is the same molecular weight as the α subunit of G proteins. Therefore, the present studies also examined the possibility of a role for G proteins in the regulation of the tracheal Cl conductance.

The inside-out conformation of patch recording allows for monitoring of Cl currents while perfusing the inner face of the membrane with putative intracellular regulatory agents. However, conflicting results— in both the areas of epithelial Cl channel regulation and that of direct G protein-regulated channel activation— have been obtained using this method. This serves as impetus for the present study, which utilizes completely different techniques.

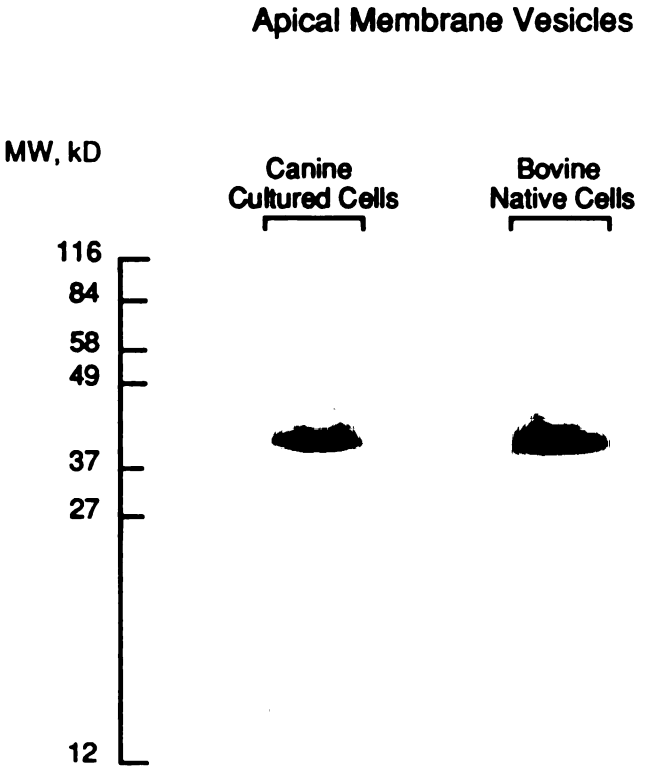

Figure 11. Autoradiogram depicting ADP ribosylation of G protein alpha subunit in apical membranes by pertussis toxin. (Courtesy C.M. Yang and J.H. Widdicombe.)

Methods

Apical membrane vesicles were prepared in a manner based on the published procedure of Langridge-Smith [1983]. The minor modifications made in this protocol and the verification of the apical origin and vesicular nature of the membranes are described in the previous chapter.

The catalytic subunit of cAMP-dependent protein kinase from bovine heart and the non-hydrolyzable GTP analogue, GTP-γ-S, were obtained from Sigma Chemical Company (St. Louis, MO).

Regulatory macromolecules were introduced by transient membrane permeabilization using hypotonic lysis (Illsley et al, 1988). While similar approaches utilizing osmotic shock have been taken in the past, the method of Illsley is the only one in which lysis and resealing have been quantitatively assessed, thus permitting the development of optimal conditions for these two events to proceed. Moreover, this technique was found superior in all respects to other previously utilized methods of macromolecular entrapment such as freeze-thaw and sonication.

Extent of lysis and resealing was quantitated by Illsley et al (1988) using fluorescent markers. Briefly, to measure the degree of lysis, vesicles were equilibrated overnight in a hypertonic ("loading") buffer containing 0.1 mM 6-carboxyfluorescein (6-CF). After washing to remove external 6-CF, the vesicles were added rapidly to either hypotonic ("lysis") buffer, or the hypertonic buffer used in overnight equilibration. The vesicles were incubated, then washed once, pelleted, and resuspended prior to assay for remaining intravesicular fluorescence. Invariably, intravesicular fluorecence was reduced to negligible levels in vesicles treated with hypotonic medium, whereas considerable levels of fluorescence were measured in vesicles added to hypertonic medium. For lysis at 25° C, with a 10 min incubation period, there was an $87 \pm 4\%$ decrease in fluorescence,

relative to the non-lysed controls.

Resealing was measured similarly using vesicles which had no fluorophore present in the overnight hypertonic incubation buffer, but were exposed to hypo- or hypertonic medium containing 6-CF. Thus, an increase in fluorescence indicates resealing; levels measured are referenced to those found in vesicles equilibrated overnight with the dye, to yield a percentage of maximal intravesicular incorporation. Dramatic increases in fluorescence (93 ± 5%, lysis/resealing at 25° C, 20 min incubation) were noted for vesicles added to hypotonic buffer, while no such effect was detectable for membranes treated with hypertonic medium.

Fluorescein-dextrans of molecular weights 40, 70 and 150 kD were used to assess the maximal molecular weight effectively entrapped using this technique. As indicated by measured fluorescence levels, fluorescein-dextrans of 40 and 70 kD were entrapped with efficiencies comparable to that of the considerably smaller 6-CF. These data justify confidence in the effective introduction of macromolecules up to 70 kD molecular weight by the hypotonic lysis procedure. Thus, the catalytic subunit of the cAMP-dependent protein kinase, which has a molecular weight of 40 kD (Nairn et al, 1985), should be entrapped using the hypotonic lysis procedure.

Lastly, Illsley et al (1988) verified the preservation of membrane sidedness and normal transport properties in vesicles subjected to hypotonic lysis. Thus, this method is ideal for the intravesicular introduction of putative transport regulators, since it, in and of itself, does not alter membrane transport.

While this technique was developed initially in placental microvillous membrane vesicles, Illsley et al (1988) characterized the optimal conditions for transient permeabilization and resealing of rabbit renal cortical and bovine tracheal epithelial apical membrane vesicles as well. The efficacies of the procedure in these two systems, as compared to placental vesicles, were ~ 60 and 100%,

respectively. The conditions specified for bovine tracheal vesicles by Illsley et al were used in these studies, and are described as follows.

Membranes were shrunken in hypertonic medium (1M KNO_3, 10 mM K-Hepes, pH 7.0) by equilibration for >24 hrs. at 4°C. The vesicles were pelleted by ultracentrifugation (60,000 x g, 10 min, 4°C) on the day of the experiment and resuspended in a small volume (200 µl) of the hypertonic incubation solution. The membranes were lysed by rapid injection into a vortexing vial of hypotonic lysis medium (20 mM K-Hepes, 5 mM $MgSO_4$, pH 7.0) containing 10 mM SPQ. The lysis medium also contained the appropriate factors required to test the actions of exogenous catalytic subunit (70-100 units of subunit/ml of total lysis reaction mixture, 25 to 500 µM ATP, 1mM Na_3VO_4, to inhibit phosphatases) or endogenous G protein (1mM GTP-γ-s, 100 µM GTP, 50 µM $MgCl_2$). These components were added immediately before use. Control conditions consisted of the identical procedure outlined above, but without any putative regulatory components added to the lysis buffer. The mixtures were allowed to stand at room temperature (~23°C) for 20 mins prior to two cycles of ultracentrifugation (60,000 x g, 10 mins., 4°C) to wash off untrapped extravesicular SPQ and regulatory factors. Wash solution consisted of cold lysis buffer with no additions. The final pellet was resuspended in a small volume (100 µl) of hypotonic lysis buffer and was kept on ice until use. Valinomycin (25 µg/mg protein final concentration) was added as an ethanolic stock to the final vesicle suspension to prevent the K permeability from being rate-limiting to Cl influx and to clamp the membrane potential to the K equilibrium potential.

External Cl influx buffers consisted of 20 mM KCl, 5 mM Tris-Hepes, 10 mM sucrose, pH 7.0). The control (0-Cl) solution had KCl substituted in an equimolar fashion by KNO_3, (J.T. Baker Co.; Phillipsburg, NJ). The influx of Cl was measured as described in the preceding chapter.

Results

Neither catalytic subunit, in the presence of ATP, nor GTP-γ-S had any effect on the initial rate of Cl influx. Values for the initial rate were as follows. In the presence of catalytic subunit, the initial rate of Cl influx was 0.10 ± 0.02 nmol.sec^{-1}.mg protein^{-1}, compared to control values of 0.13 ± 0.00 nmol.sec^{-1}.mg protein^{-1}. When GTP-γ-S was added in the lysis medium, the initial rate was 0.09 ± 0.01 nmol.sec^{-1}.mg protein^{-1}. This is not different from the control value for initial rate, which was 0.09 ± 0.01 nmol.sec^{-1}.mg protein^{-1}.

Discussion

Studies utilizing single channel measurements from several laboratories strongly suggest that the apical membrane Cl channels of tracheal epithelial cells are subject to regulation by the cAMP-dependent protein kinase. As these observations, which were made on isolated patches of membrane using exogenous catalytic subunit of protein kinase A (in the presence of ATP), show elevation of channel activity, the phosphorylation of a membrane-bound component that directly or indirectly gates the Cl channel is implied. Thus, it seemed that entrapment of the catalytic subunit and ATP into apical membrane vesicles and monitoring the Cl influx rates with SPQ fluorescence would be an ideal alternative means of approaching the question of how the channel is regulated by phosphorylation. However, no effect of this combination of factors was noted.

Schoumacher et al, (1987) noted that the extent of channel activation in excised patches after addition of catalytic subunit and ATP was rather variable. Implied here, then, is the idea that differing phosphatase levels may be the cause of this scatter. The conditions (1mM Na_3V0_4, an inhibitor of phosphatase activity) of the present study were chosen to minimize such an effect, and therefore maximize any change in the signal. Thus, the lack of effect should not be due to excessive phosphatase activity.

Alternatively, the variability in activation observed by Schoumacher et al could be interpreted as reason to believe that another patch-associated cell component may be required for kinase A action to proceed. Given such a schema, and realizing the difficulty in controlling for the association and levels of extraneous cell contents with inside-out patches, it is not unimaginable that such variation in the data was observed. In light of the existing evidence, this seems more probable than the possibility—evoked by the negative results of the present

study—that protein kinase A has no role at all in the regulation of Cl channel activity.

Thus, the lack of effect of catalytic subunit on Cl transport in apical membrane vesicles can be explained by the idea that at least another cell component is required to engage kinase activity. Preparation of vesicles entails more rigorous mechanical and chemical disruption than patch formation. The procedure, then, conceivably could remove the factor needed by kinase A to gate the Cl channel. This is more feasible an explanation than the possibility that the catalytic subunit and/or SPQ were not entrapped since there is reasonable confidence in the effectiveness of hypotonic lysis in sequestering these molecules into vesicles. Illsley et al (1988) have demonstrated the efficacy of hypotonic lysis in the intravesicular incorporation of macromolecules significantly larger than the catalytic subunit (40 kD); unrestricted entry of molecules with masses up to 70 kD has been reported. Moreover, the SPQ signal was sufficient and comparable to levels achieved with overnight incubation, demonstrating that resealing was adequate.

Similarly, all of the above arguments would hold for a situation in which cell detached patches have a variable degree of association of a kinase inhibitor. The possibility exists that such a putative inhibitor may remain in the membrane vesicles used in this study, and thus would prevent activation of the entrapped catalytic subunit.

Taken together, these observations warrant further investigation using independent techniques. Particular emphasis should be placed on determining whether some intracellular factor—associated strongly enough with the apical membrane to be excised along with an inside-out patch—is neccessary for the stimulation of channel activity by kinase A.

Direct regulation/gating of ion channels by G proteins has been reported for

atrial myocytes (Yatani et al., 1987; Logothetis et al, 1987; Codina et al, 1987; Breitwieser and Szabo, 1985; Pfaffinger et al., 1985) as well as epithelial cells of the kidney (Light, Ausiello, and Stanton, submitted) using single channel and whole cell recording techniques. The SPQ fluorescence quench assay and the hypotonic lysis and entrapment technique were used in apical membrane vesicles from bovine tracheal epithelial cells to investigate the possibility that tracheal Cl channels are gated by direct G protein interactions. There was no difference in initial influx rate in control vesicles and those into which the non hydrolyzable GTP analogue, GTP-γ-S, had been entrapped. Several different interpretations can be drawn from this negative result.

Despite the results of these studies, direct G protein regulation may still be relevant to apical Cl channel stimulation. The experiments here assume that apical membrane vesicles have endogenous G proteins, which, in the presence of excess amounts of GTP (GTP-γ-S), are capable of activating the apical Cl channel. An effect may not be detectable in the system used in these studies if the following model applies for epithelial G protein/channel activation.

Normal agonist action is due, presumably, to binding of that agent to a basolaterally localized receptor. The activated complex, which is restricted to the serosal membrane, thus in turn stimulates a G protein in the same membrane. For direct G protein/Cl channel interactions to proceed, the activated G complex must somehow have access to the apical membrane. Though the tight junctions restrict movement of integral membrane proteins which span the entire plasma membrane, it is possible that a G protein associated exclusively with the inner leaflet would be able to diffuse and have free access to the apical aspect of the epithelial cell.

G proteins are a large family of molecules (Bourne, 1989). The involvement, in channel regulation, of a G protein different from the PTX-sensitive,

ADP-ribosylated G_i has not been ruled out by these studies. While most of the G proteins that thus far have been shown to regulate ion channels directly appear to resemble G_t, this has not yet been established as a general characteristic. Thus, a G protein different from G_i and localized to the basolateral membrane could be activated as described in the preceding model, and conceivably could gate the apical Cl channel directly.

Summary

The hypotonic lysis procedure of Illsley et al [1988] was used to entrap either the catalytic subunit of cAMP-dependent protein kinase or the non-hydrolyzable GTP analogue, GTP-γ-S, along with their respective cofactors, into bovine tracheal epithelial cell apical membrane vesicles prepared as previously described (Langridge-Smith et al, 1983; Fong et al, 1988). Initial rates of Cl transport (J_{Cl}) were monitored with the Cl-sensitive fluorescent dye, 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ; Illsley and Verkman, 1987). J_{Cl} in control vesicles was compared to the rates in vesicles to which catalytic subunit or GTP-γ-S had been introduced. With 20 mM Cl gradients, a membrane potential of 0 mV, and at 37 °C, the mean J_{Cl} under control conditions was 0.109 nmol. sec^{-1}.mg $protein^{-1}$. Neither the addition of catalytic subunit nor GTP-γ-S influenced J_{Cl} significantly (values given as per cent change from control); for catalytic subunit, the change in J_{Cl} was - 17±12%, while GTP-γ-S caused a -1 ±14% alteration in J_{Cl}. While these data argue against models in which direct modulation of channel activity by either catalytic subunit or G proteins plays a major role, they do not exclude more complex regulatory relationships between these agents and the channel.

Chapter Four

Cl Uptake by Na-coupled Basolateral Cotransport

The mechanism of basolateral Cl uptake by a Na-coupled cotransport system has been examined using a number of different approaches and experimental preparations by several groups. However, the conclusions from these studies have been in disagreement. On the basis of data from oxygen consumption studies, Welsh (1984) argued that the mechanism of basolateral Cl uptake was K-, as well as Na-, dependent. Using tracer uptake measurements on dispersed cells from canine trachea, Widdicombe et al (1981, 1983) found no evidence of such a K requirement for cotransport. Working with a dispersed bovine tracheal epithelial cell preparation, these results recently have been confirmed by Musch and Field (1989).

The following studies were undertaken in order to determine which cotransporter is relevant in the transepithelial secretion of Cl. Thus, the preparation must maintain the anatomical and physiological polarity of the original tissue; it must clearly segregate the apical and basolateral membrane domains, as well as maintain the ability to transport vectorially. To this end, confluent, electrically tight monolayers of canine tracheal epithelial cells grown on thin, permeable supports have been used in these studies. This preparation retains the directionality of the original tissue, and has been demonstrated previously to have vectorial ion transport processes resembling those of the freshly dissected tracheal epithelium (Coleman et al 1984). Because the monolayers are grown on very thin (~ 10 μm) filters, the serosal dead space is minimized. Thus, this preparation permits the practical study, with radioactive tracer techniques, of tissues possessing the properties of the native epithelium. Taken together, the results of these experiments specifically determine the K dependency of the Cl uptake mechanism important in directional transport.

Introduction

The model for tracheal electrolyte secretion that is widely accepted is one in which Na and Cl are taken up by the cell from the blood via an electroneutral cotransporter (Frizzell et al, 1979). The energy in the Na gradient maintained by the basolaterally situated Na/K ATPase permits this uptake. Chloride thus is accumulated in the cell to a level that is sufficient to drive its conductive exit across the apical membrane and into the lumen of the trachea. Obligatory movement of Na and water ensures net electroneutrality and isosmotic secretion.

Many other epithelial and non-epithelial tissues maximize the Cl transporting potential of the Na gradient by coupling the transfer of two Cl ions to that of one Na and one K (Geck et al 1980, Greger 1981, Oberleithner 1983). In these systems, the K that enters then leaves via a conductive pathway. There has been some question as to whether such a K dependence of the cotransporter exists in tracheal epithelial cells. This question forms the basis for undertaking the study presented here. We have approached the problem using two different transport assays: I_{sc} measurements in Ussing chambers, and tracer uptake measurements. The results of our experiments provide evidence for a K-dependent cotransport mechanism in primary cultures of dog tracheal epithelium.

Methods

Tissue Culture of Electrically Tight Confluent Monolayers of Tracheal Epithelial Cells

Primary cultures of dog tracheal epithelial cells were obtained by the protease digestion of Wu (1985) and grown on collagen coated polycarbonate filters (Coleman et al, 1984). Trachae were removed from dogs immediately after death and washed with cold phosphate buffered saline (PBS). The tracheae were split open by a longitudinal incision through the cartilage, and pinned out. Shallow longitudinal cuts about 1/4 inch apart were made in the mucosa. The mucosal strips were pulled off with a pair of haemostats and washed in PBS containing 5 mM dithiothreitol (DTT) to remove adherent mucous. After 3 more rinses in this solution, the strips were washed in cold PBS to dilute out any remaining DTT, and transferred to a 50 ml tube containing a 0.05% protease/PBS solution (Sigma Chemical Co., St. Louis, MO). This was left overnight at 4°C; protease activity was stopped the next morning by the addition of 10% bovine serum albumin. The mucosal strips were gently agitated to release cell clumps by repeated inversion of the tube for 20 secs. Connective tissue strips were removed and the remaining cells were pelleted by centrifugation (120 x g, 10 mins.) with a table top centrifuge (Model HN-SII, International Equipment Company, Needham Hts, MA). The pellet was resuspended in an appropriate volume of a 1:1 mixture of Dulbecco's Modified Eagles Medium and Ham's F-12 containing pen-strep, fungizone, gentamycin, non-essential amino acids, and 5% fetal calf serum. Cells were grown in 35 mm 6-well plates on either 25 mm Nucleopore filters or on 25 mm Costar polycarbonate membrane inserts coated with human placental collagen (Sigma Chemical Company, St. Louis, MO) at a density of 2.5×10^5 cells/cm^2. Monolayers were confluent after 5 days and studied on days 7-20; the electrical

properties of these monolayers remained constant within this period.

Electrical Measurements

Short-circuit current (I_{sc}) measurements were performed using a conventional Ussing chamber apparatus. The chambers were filled with HCO_3-buffered Krebs Henseleit solution (pH = 7.4) on both sides, maintained at 37°C, and bubbled continuously with 95% O_2/5% CO_2. The transepithelial p.d. was maintained at zero with a voltage clamp (model 742 C, Bioengineering, the University of Iowa), and the current required (the short-circuit current, I_{sc}) was monitored continuously on a pen recorder. Current pulses of 200 msec duration were applied every 20 sec to displace the transepithelial voltage from 0 to 0.5 mV. The transepithelial resistance was then calculated from the size of the current and voltage deflections. Only tissues with R>100 $\Omega.cm^2$ were used. The experimental protocol was as follows. Once I_{sc} stabilized, 10^{-4} M amiloride (Merck, Sharp, and Dohme, West Point, PA) was added to the mucosal chamber to abolish any component of the I_{sc} attributable to Na transport. At this point, isoproterenol (10^{-5} M) was applied both mucosally and serosally to stimulate Cl secretion. Barium, as either $BaCl_2$ or $Ba(NO_3)_2$, was added on the serosal side in a cumulative fashion as 1:100 or greater dilutions of 0.1 M stock solutions. The NO_3 salt of Ba^{++} was tested in order to control for possible transport effects of the small amounts of Cl introduced as $BaCl_2$. Doses higher than 5×10^{-3} M did not further lower I_{sc} significantly. After the last addition of barium, serosal exposure to 10^{-4} M ouabain brought the I_{sc} to zero. Isoproterenol, $BaCl_2$, and ouabain were all supplied by Sigma Chemical Co. (St. Louis, MO). $Ba(NO_3)_2$ was from Aldrich Chemical Co. (Milwaukee, WI).

Radioactive Tracer Flux Measurements

^{86}Rb was obtained from Amersham (Arlington Heights, IL). Uptake media generally consisted of Krebs Henseleit with a final ^{86}Rb concentration of 0.5 μCi/ml. The monolayers were preincubated in Krebs Henseleit solution for 30 minutes. Amiloride (10^{-4} M) was added during the preincubation period (at t = -20 min) to abolish Na absorption. For experiments testing the effects of bumetanide (10^{-4} M; Hoffman LaRoche, Nutley, N.J.), this inhibitor was added along with the amiloride. Chloride secretion was stimulated by the addition of isoproterenol (10^{-5} M) at t =-5 min. At time 0, monolayers were introduced to the uptake media ± 10^{-4} M bumetanide and were removed at desired time points. In some experiments, Hepes buffered 0-Cl and 0-Na solutions were used, and the control Krebs solution buffer was changed from HCO_3 to Hepes as well. The compositions of these modifications of the standard HCO_3-buffered Krebs Henseleit solution are given in Table 3. In all cases, the preincubation and uptake solutions were maintained at 37°C in a Precision (model 67100, Precision Scientific Group, Chicago, Il) water bath. Following uptake, the filters were washed in a large volume (200 ml) of tracer-free Krebs Henseleit solution (± 10^{-4} M bumetanide) at 4° C for 15 seconds to remove extracellular ^{86}Rb, then transferred to scintillation vials. Control experiments in which monolayers were equilibrated with ^{3}H-mannitol (New England Nuclear, Boston, MA) in addition to ^{86}Rb and washed for varying lengths of time indicated that a 15 second wash sufficiently removed extracellular uptake medium without also altering intracellular ion contents (data not shown). Prior to counting, tissue protein was dissolved by overnight exposure of the filters to 2 ml of 0.1 N NaOH. A 100 μl sample was taken for protein assay using the method of Smith et al (1985). There was little variation in protein content from monolayer to monolayer. Safety-Solve scintillation cocktail (RPI, Mount Prospect, Il) was added to the remaining samples, which were then

Table 3

Compositions of Uptake Solutions
(mM)

Solution	Major Constituents
Control Hepes-buffered	120 mM NaCl 25 mM Na-Hepes 3.8 mM KCl
0-Cl Hepes-buffered	125 mM Na Isethionate 20 mM Na-Hepes 3.8 mM K-Hepes
0-Na Hepes-buffered	120 mM NMG-Cl 25 mM NMG-Hepes 3.8 mM KCl

In addition, all solutions contained:

1.2 mM KH_2PO_4
5.9 mM Glucose
2.5 mM $CaSO_4$
1.2 mM $MgSO_4$

counted on a Beckman beta counter (model LS7500; Beckman Instruments, Irvine, CA).

Tissues grown on Nucleopore filters had both apical and basolateral sides exposed to the ^{86}Rb containing solution. Those tissues cultured on Costar inserts received tracer on only one side. This enabled us to control for any uptake attributable to possible apical K transport. The results were qualitatively the same for experiments using tissues grown on Nucleopore filters and those in which only basolateral uptake was studied. Percent inhibition by bumetanide was more pronounced in the latter experiments.

Results

Contribution of basolateral g_k to I_{sc}

In a consideration of the role of the basolateral membrane in transepithelial Cl secretion, it can be noted that the fraction of maximal Ba^{++}-inhibitable Cl secretion differs for Na/Cl and Na/K/2Cl cotransporters (Fig.12). The case of Na/Cl cotransport is depicted on the left side of the figure. Here, to maintain electroneutrality, the movement of six Cl ions across the basolateral membrane and into the cell must be accompanied by the exit of six positively charged species across the same membrane. For an Na/Cl cotransporter, this balancing function can be ascribed to the exit of four K ions across g_k, and two Na ions via the Na/K ATPase. In the case of an Na/K/2Cl carrier, however, five K ions exit via g_k whereas only one unit of positive charge— one Na ion— is moved out of the cell via the action of the pump. Thus, in the case of Na/Cl cotransport, inhibition of g_k would attenuate two-thirds or 66.7% of Cl secretion by the epithelium. If Cl secretion proceeds as a result of a K-dependent basolateral Cl uptake mechanism, five-sixths or 83.3% of the net secretion would be inhibited by blockage of g_k.

A typical experiment testing the effect of serosal Ba^{++} on I_{sc} is depicted in figure 13a. Eadie-Hofstee analysis of the data showed that there are at least two binding sites for Ba^{++}. The low affinity binding site (Fig. 13b) has an apparent K_d of $1x10^{-4}$M while the corresponding value for the high affinity binding site is $2x10^{-6}$ M. The Ba^{++}-sensitive fraction of the I_{sc} was determined by taking the ratio:

$$\frac{(I_{sc} \text{ before } Ba^{++} - I_{sc} \text{ after } Ba^{++})}{(I_{sc} \text{ before } Ba^{++} - I_{sc} \text{ after } 10^{-4} \text{ M ouabain})}$$

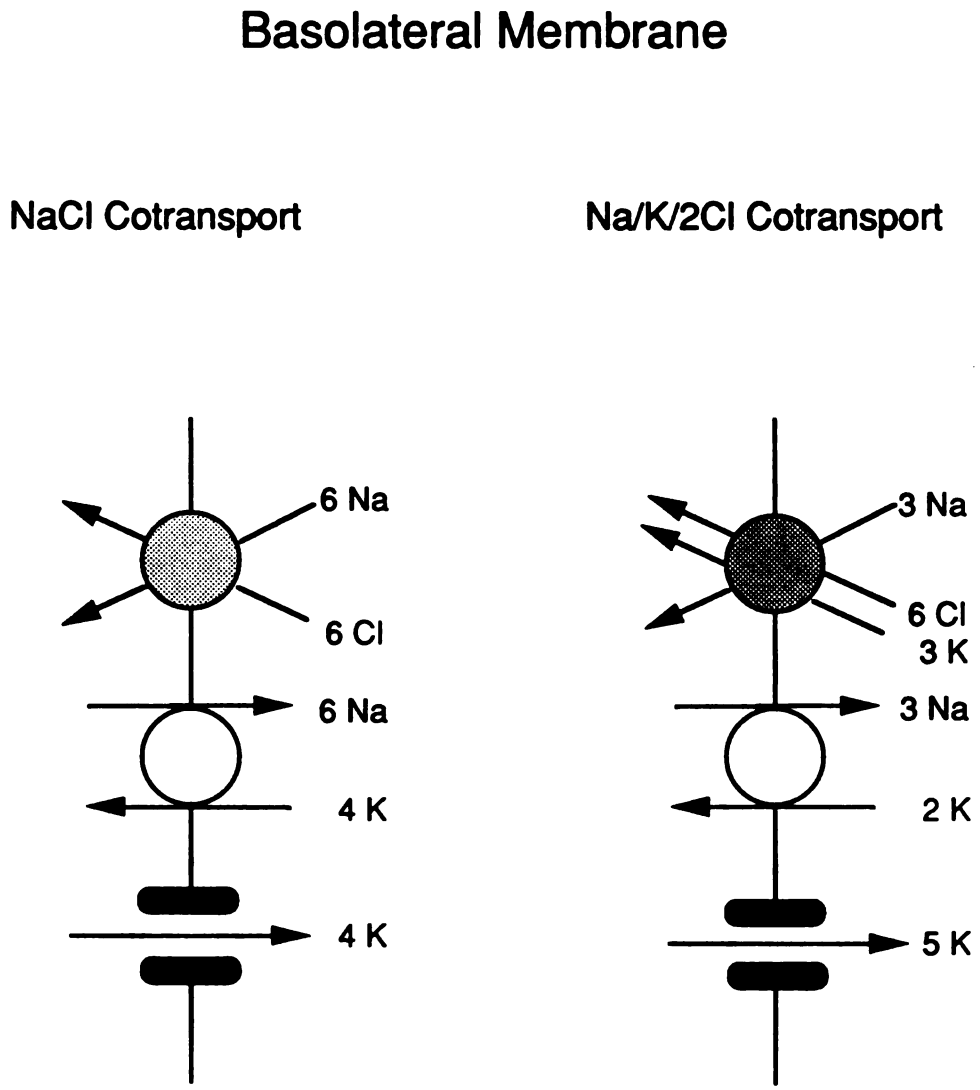

Figure 12. Net movements of Na, K, and Cl across basolateral membrane of Cl-secreting epithelia with NaCl or Na/K/2Cl cotransport mechanisms. Analysis given in text.

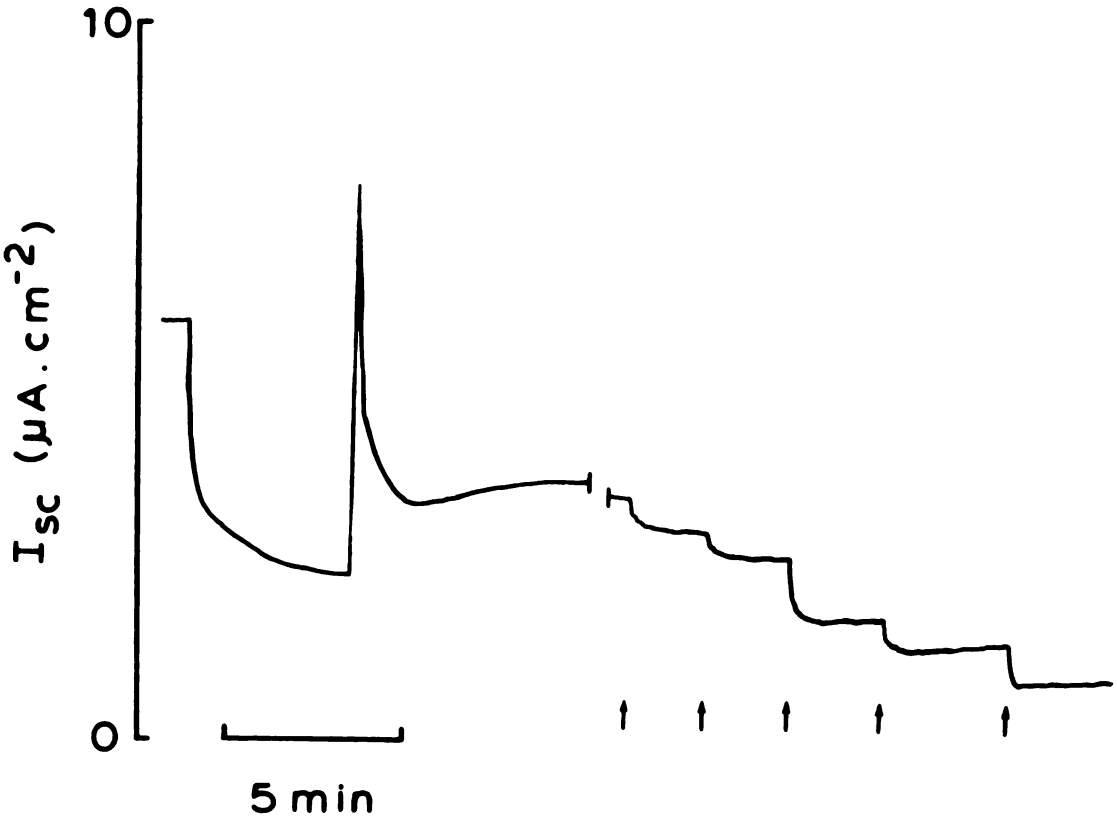

Figure 13a: Short-circuit current (I_{sc}) record depicting inhibition by cumulative doses of serosally applied Ba^{++} to a monolayer of canine tracheal epithelial cells. Tissue was pretreated with amiloride (10^{-4} M) mucosally to eliminate the component of I_{sc} due to Na absorption (first arrow). Chloride secretion was stimulated by application of isoproterenol (10^{-5} M) serosally and mucosally (second arrow). After stabilization of I_{sc}, Ba^{++} was added in the following doses: 10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3} M (arrows at step changes).

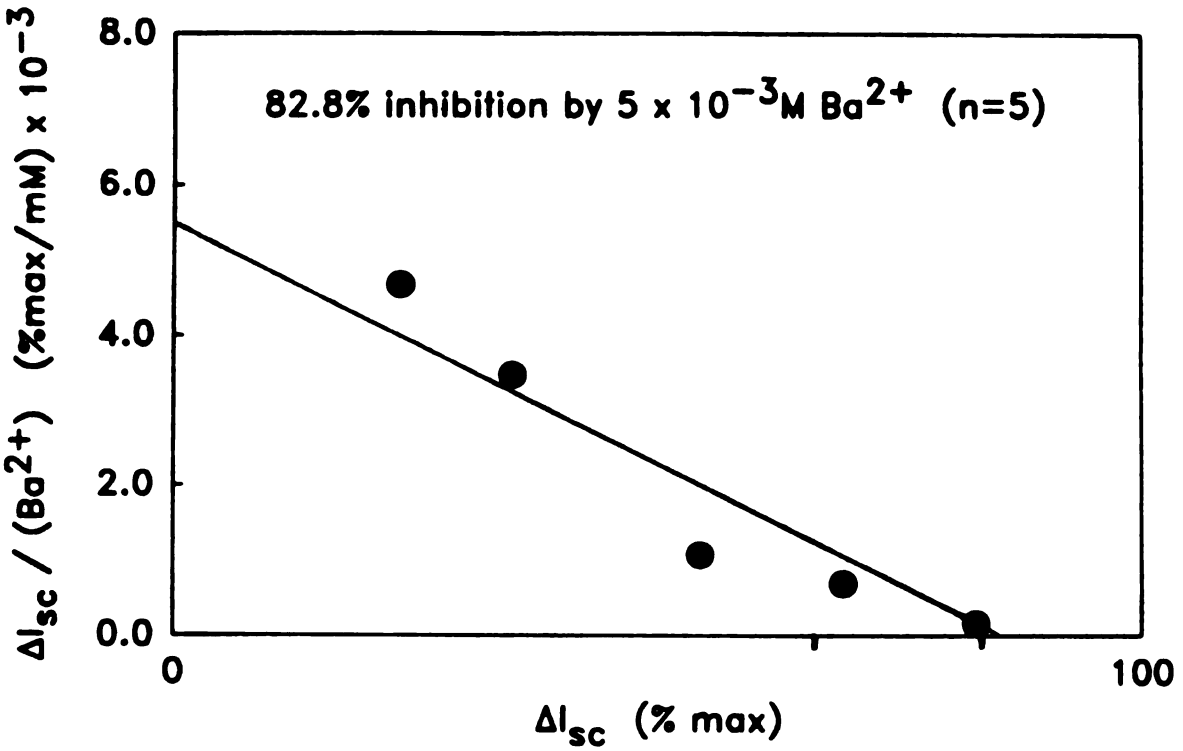

Figure 13b. Eadie-Hofstee plot of Ba^{++} inhibition data. Figure shows low affinity site for inhibition with K_d of 1 x 10^{-4} M.

After maximal (5 X 10^{-3} M) doses of Ba^{++}, as can be seen in the record shown in figure 13, this fraction was about five-sixths of the total I_{sc} attributable to chloride secretion. This effect was highly reproducible (82.8 ± 0.0%, n=5). The inhibition data for the low affinity site can be extrapolated to the x-intercept by linear regression, thus giving the maximal percentage of inhibition by Ba^{++}. This value was 83.0 ± 4.1% (mean ± SEM, n=8, $p<0.01$, see figure 13). This is significantly different from the 66.7% expected to be inhibited by Ba^{++} in the case in which basolateral Cl uptake is dependent solely on the presence of Na (NaCl cotransport). Conversely, 83.0% is in close agreement with the 83.3% inhibition predicted if basolateral Cl uptake proceeds via an Na/K/2Cl cotransporter. There was no difference between $BaCl_2$ and $Ba(NO_3)_2$ in reducing I_{sc}. Table 4 summarizes the data from these experiments.

Dependence of ^{86}Rb uptake on Na and Cl

Influxes of ^{86}Rb were first studied with Nucleopore filters exposed on both sides to tracer. Fig. 14 shows J_{Rb} was bumetanide sensitive. In order to determine the sidedness of the uptake mechanism, monolayers grown on Costar inserts were used. Paired experiments in which ^{86}Rb addition was confined to either the apical or the basolateral membrane demonstrate that the pathway of tracer accumulation is limited to the basolateral membrane: influx of ^{86}Rb from the apical medium was negligible (0.23 ± 0.03 nEq.cm^{-2} after 2 mins.; n=4), with no bumetanide-sensitivity, whereas basolateral application resulted in significant, time-dependent uptake (6.78 ± 0.33 nEq.cm^{-2} after 2 mins.; n=4; Fig. 15). Figure 16 shows inhibition of the initial rate of tracer uptake by the serosal application of bumetanide (10^{-4} M). Regression analysis estimates the extent of inhibition to be 52 ± 10% (n=4).

Table 4

Short-circuit Current Changes in Response to Basolateral Ba^{++} Application

$[Ba^{++}]$	% Change in I_{sc}
1×10^{-6} M	4.1 ± 1.2%, n=8
5×10^{-6} M	5.6 ± 2.0%, n=5
1×10^{-5} M	10.8 ± 1.9%, n=9
5×10^{-5} M	23.4 ± 2.9%, n=5
1×10^{-4} M	34.8 ± 2.6%, n=9
5×10^{-4} M	54.2 ± 6.7%, n=6
1×10^{-3} M	69.1 ± 3.5, n=10
5×10^{-3} M	82.8 ± 4.2%, n=5

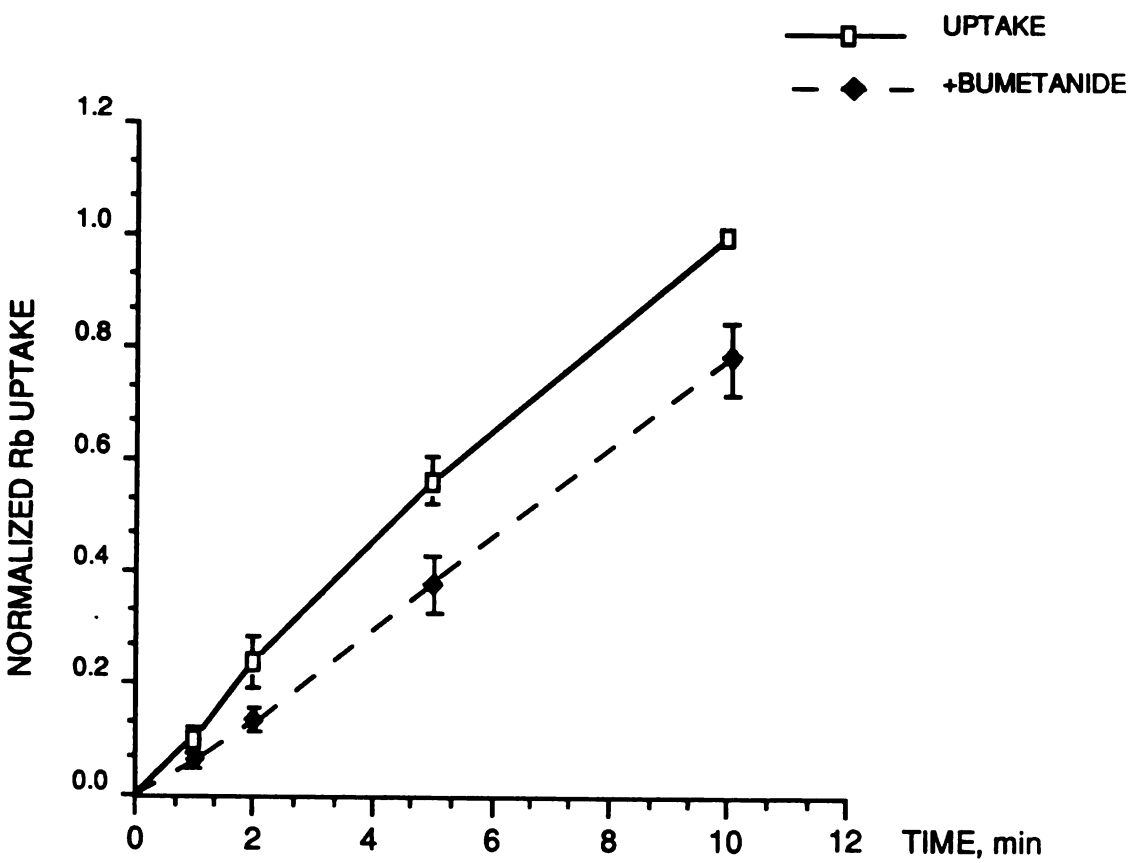

Figure 14. Time-dependent uptake of ^{86}Rb by tracheal monolayers grown on Nucleopore filters. Bumetanide (10^{-4}M) inhibition of initial rate illustrated is ~32%. Data normalized to control 10 minute uptake, 205.1 +/- 33.77 nEq/filter (mean +/- SEM, n=6).

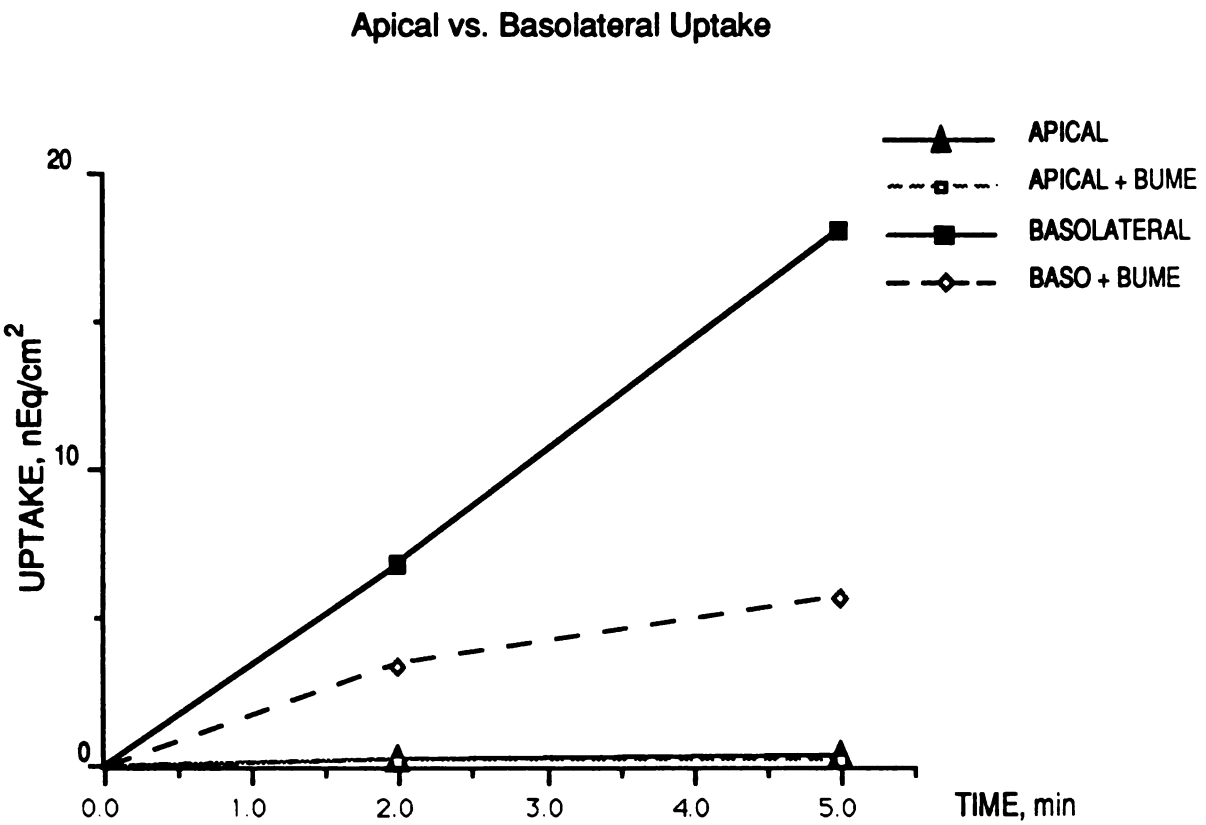

Figure 15. Time-dependence and bumetanide sensitivity of ^{86}Rb uptake from basolateral side of tissue vs. the same from the apical aspect. Apparent here is the absence of either relationship in the latter case. Association of tracer to the tissue when introduced apically is minimal. Error bars are smaller than the symbols used. For all points, n=4.

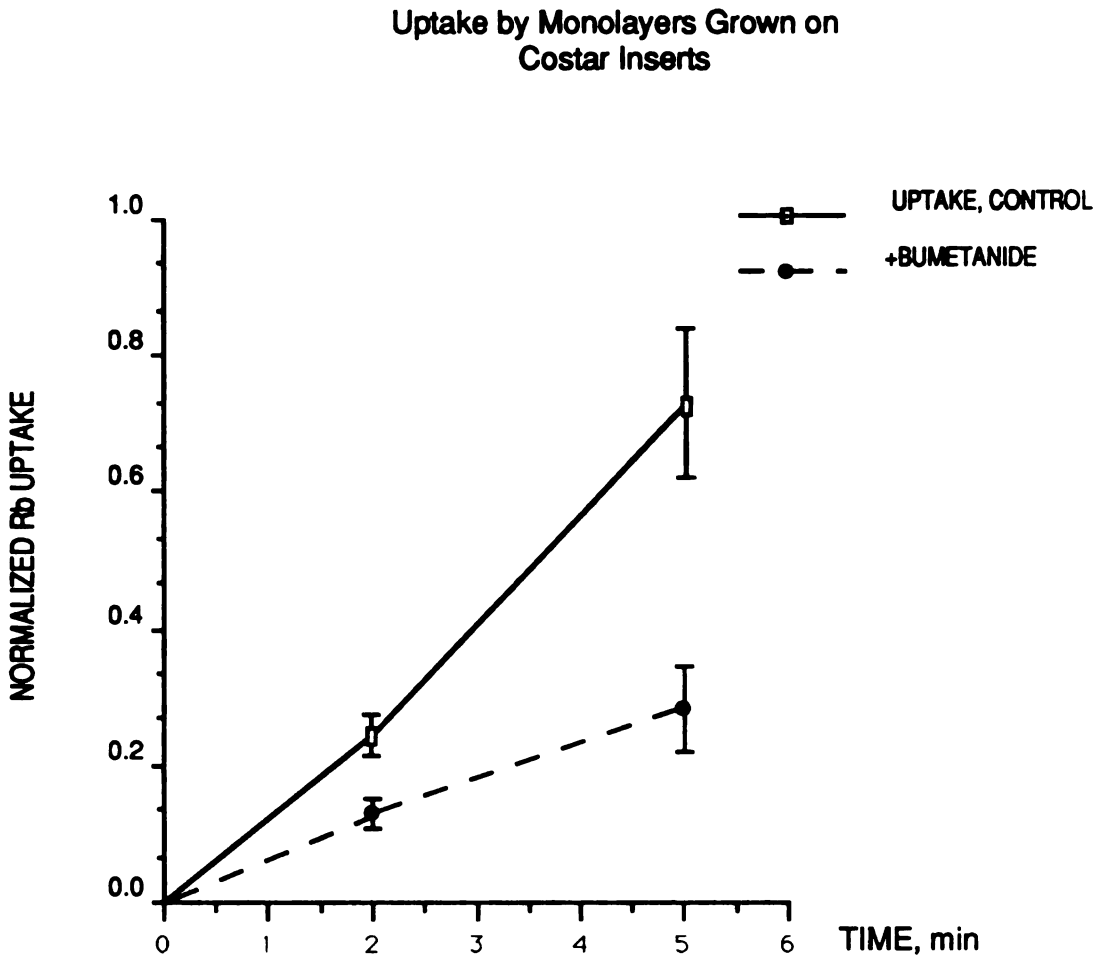

Figure 16. Uptake of ^{86}Rb by tracheal monolayers grown on Costar inserts as a function of time. Inhibition of initial rate of uptake by 10^{-4} M bumetanide is ~52%. Data normalized to control 10 min uptake, 132.18 +/- 12.18 nEq/filter (mean +/- SEM, n=5).

Figure 17 summarizes the results of experiments designed to test ion dependencies of the bumetanide-sensitive ^{86}Rb uptake. Here, ^{86}Rb uptake was measured at 5 minutes for all conditions; the data represent paired comparisons for monolayers grown from the same tracheae, tested on the same day. Uptake medium here was completely Cl- or Na- free and Hepes buffered (see table 1). At 5 min, uptake under control conditions was 23.9 ± 2.7 nEq.cm^{-2} (n=7) while application of bumetanide lowered uptake to 7.5 ± 0.9 nEq.cm^{-2} (n=7). Inhibition of uptake caused by total Cl replacement was not increased further by the addition of bumetanide; in the former, ^{86}Rb uptake at 5 min was 15.1 ± 1.8 nEq.cm^{-2} (n=7), as compared to 13.8 ± 1.0 nEq.cm^{-2} (n=7) for the latter case. Thus, uptake was reduced by removal of Cl and bumetanide sensitivity was lost in 0-Cl medium. Similar experiments using N-methyl-D-glucamine as a Na substitute demonstrate that the addition of bumetanide did not decrease ^{86}Rb uptake further. For NMG substitution, uptake was 3.6 ± 0.6 nEq.cm^{-2} (n=7), while bumetanide addition, under conditions of total Na replacement, yielded an uptake of 2.8 ± 0.6 nEq.cm^{-2} (n=7, $p>0.05$). Interestingly, the fluxes observed in the Na substitution experiments were lower than those obtained from replacement of Cl. To test the hypothesis that this effect is due to the reduction of pump-mediated ^{86}Rb (K) uptake, we compared the effect of Na replacement with that of basolateral ouabain (10^{-4} M) addition. Application of ouabain would eliminate totally the portion of ^{86}Rb influx attributable to ATPase activity. For tissues from the same preparation, control uptake of ^{86}Rb was 14.2 nEq.cm^{-2}. Application of bumetanide reduced uptake to 5.8 nEq.cm^{-2}, while bumetanide addition under conditions of either Na substitution or ouabain treatment lowered uptake levels to 2.2 nEq.cm^{-2} and 0.4 nEq.cm^{-2}, respectively. The apparently incomplete inhibition of influx by NMG substitution could be due to residual intracellular Na or increased uptake through the basolateral K conductance. Since removal of external Na should

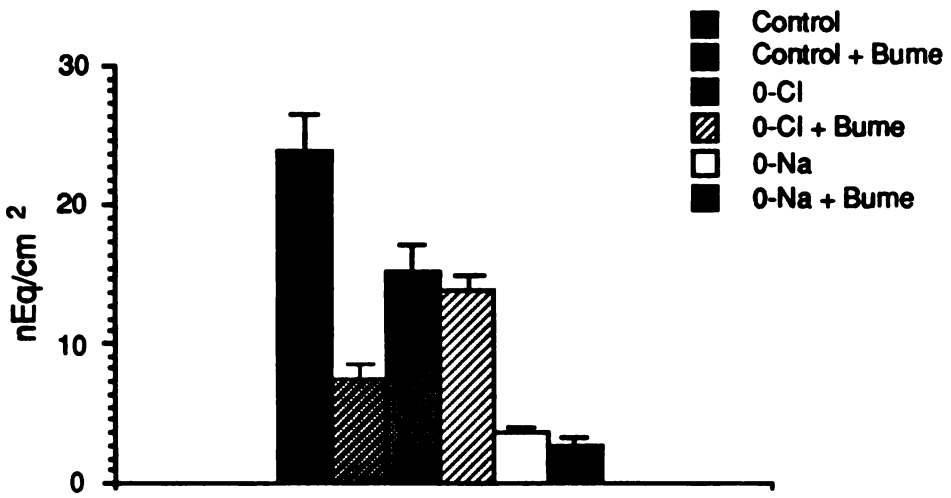

Figure 17. Five minute uptakes of ^{86}Rb by tracheal monolayers grown on Costar inserts under the following conditions: control +/- bumetanide, 0-Cl +/- bumetanide, and 0-Na +/- bumetanide. See text for further discussion. In all cases, n=7 monolayers.

cause basolateral membrane hyperpolarization, the entry of ^{86}Rb into the cell is favored. Ouabain-poisoned tissues, on the other hand, should be markedly depolarized. Taken together, these observations indicate that the lower absolute level of ^{86}Rb uptake observed under conditions of complete Na replacement by NMG is due to suppression of Na/K ATPase activity.

In order to control for changes in ouabain-sensitive ^{86}Rb uptake, the Na- and Cl-replacement experiments were repeated in ouabain-poisoned tissues (1hr, 10^{-4} M). Time-dependent uptake of ^{86}Rb was preserved in ouabain-poisoned tissues (fig. 18). In such tissues, ouabain-insensitive ^{86}Rb uptake at 5 min in 0-Cl was not different from that in 0-Na (fig. 19). Bumetanide inhibited ^{86}Rb uptake by ~50% in control but not in Na-free or Cl-free media.

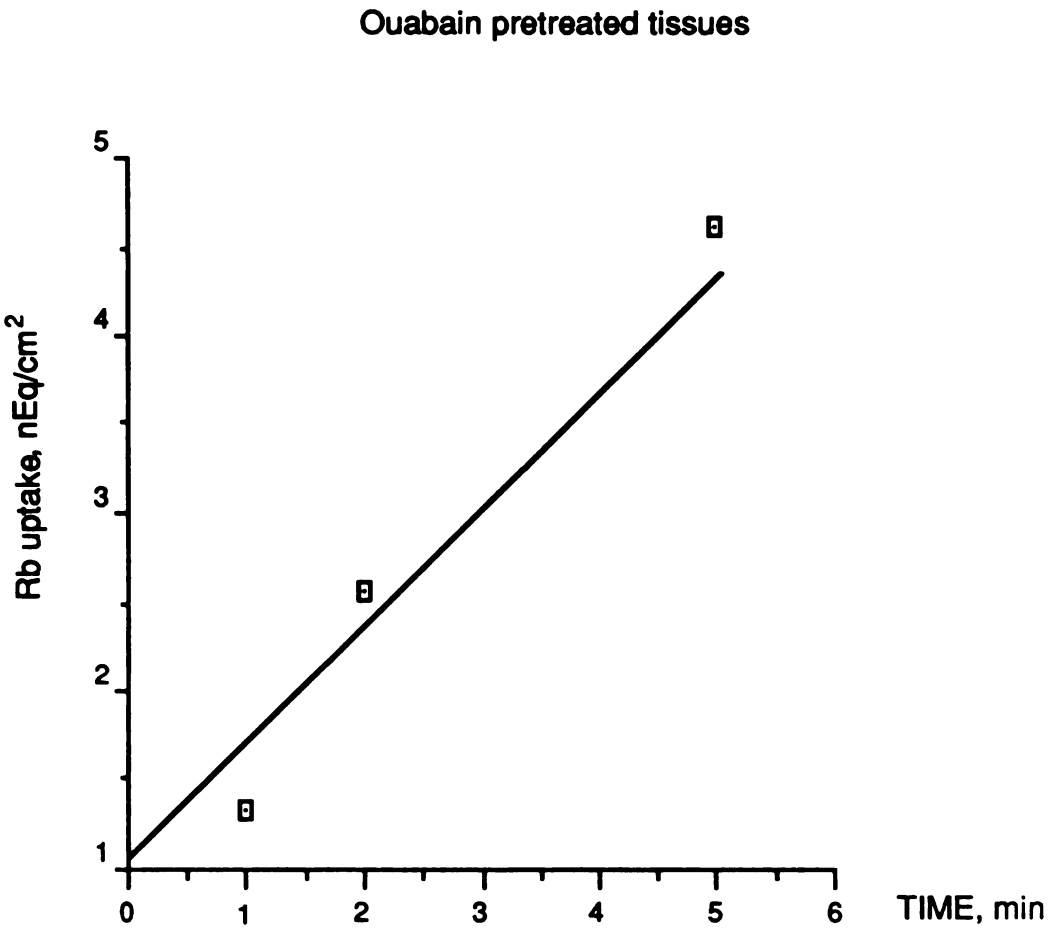

Figure 18. Time-dependent uptake of ^{86}Rb persists after pretreatment with 10^{-4}M ouabain.

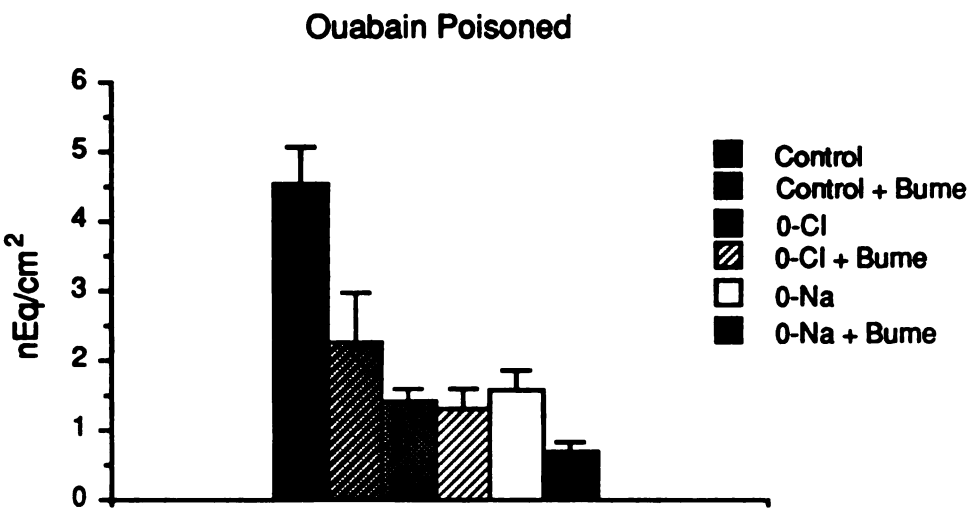

Figure 19. Five minute uptakes of ^{86}Rb by tracheal monolayers, pretreated with 10^{-4} M ouabain, in control, 0-Cl, and and 0-Na solutions +/- bumetanide. For control case, n=3; in all other conditions, n=2.

Discussion

Increasing the Cl transporting efficiency of the Na gradient by coupling the movement of two Cl ions to the transfer of one Na ion has been discussed previously by others (Welsh, 1984; Epstein and Silva, 1985) The coupling of one K ion to the process constitutes an energetically favorable means of achieving electroneutral cotransport. The present study provides evidence for K dependence of the basolateral cotransporter in primary cultures of canine tracheal epithelium using two completely different approaches.

The Ba^{++}-inhibitable I_{sc} is consistent with that expected for a K-dependent basolateral cotransporter

I_{sc} was monitored after amiloride abolition of Na absorption and isoproterenol stimulation of Cl secretion. The basolateral g_k was blocked with Ba^{++}, in the forms of its Cl and NO_3 salts. By determining the maximal Ba^{++}-inhibitable I_{sc}, the K ion dependency of the basolateral cotransporter can be resolved as detailed by the analysis given in the Results section. The maximal inhibition was found to be the same for both $BaCl_2$ and $Ba(NO_3)_2$. The results of the Ussing chamber studies, in which the maximal inhibition by serosal Ba^{++} is approximately five-sixths of the I_{sc}, are consistent with a K-dependent Cl uptake pathway. Maximal Ba^{++} inhibition of I_{sc} in each tissue was determined by using a cumulative dose-response protocol. Thus further insight into the mechanism of Ba^{++} blockage of the basolateral g_k was obtained from Eadie-Hofstee analysis. At least two inhibitory binding sites—high and low affinity—are suggested by our data. Thus, the presence of two types of K channels is implicated.

Uptake of ^{86}Rb is inhibited by basolateral bumetanide and is dependent on the presence of Cl and Na

The activity of the basolateral cotransporter would depend on the presence of all transported species, and would be sensitive to loop diuretics such as bumetanide and furosemide. Following this line of thought, Dharmasathaphorn et al (1985) obtained evidence for basolateral Na/K/2Cl cotransport in confluent monolayers of T_{84} cells by measuring uptakes of ^{86}Rb, ^{36}Cl, and ^{22}Na. These were interdependent and all sensitive to basolateral bumetanide.

The effects of bumetanide addition, as well as replacement of basolateral Cl or Na, on uptake of ^{86}Rb, a tracer for K transport, were first evaluated. The data demonstrate considerable inhibition by all of the above-mentioned maneuvers. Moreover, addition of bumetanide to uptake media from which Cl or Na were eliminated did not result in an additive inhibition. Therefore, this suggests that ^{86}Rb uptake is linked to bumetanide-sensitive transport of both Cl and Na.

The percentage inhibition of ^{86}Rb uptake by bumetanide (~50%) is consistent with the value predicted using information drawn from the literature, and assuming that the mechanism of basolateral Cl entry is via an Na/K/2Cl cotransporter. Figure 20 shows the relative magnitudes of basolateral ^{86}Rb influx via the Na/K ATPase, the Na/K/2Cl cotransporter, and the K conductance. Thus, for every 3 K ions entering the cell on the cotransporter, 2 K ions must enter via the ATPase. These 5 K ions must leave the cell by means of the K conductance; the difference between the unidirectional fluxes through the K channel must be 5 ($J_{io} - J_{oi} = 5$). If the movement of K through the conductance is determined by simple diffusion, then the ratio of the efflux (J_{io}) to the influx (J_{oi}) can be calculated by applying the Ussing flux ratio equation:

$$J_{io}/J_{oi} = aK_i/aK_o\, e^{EF/RT} \quad (7)$$

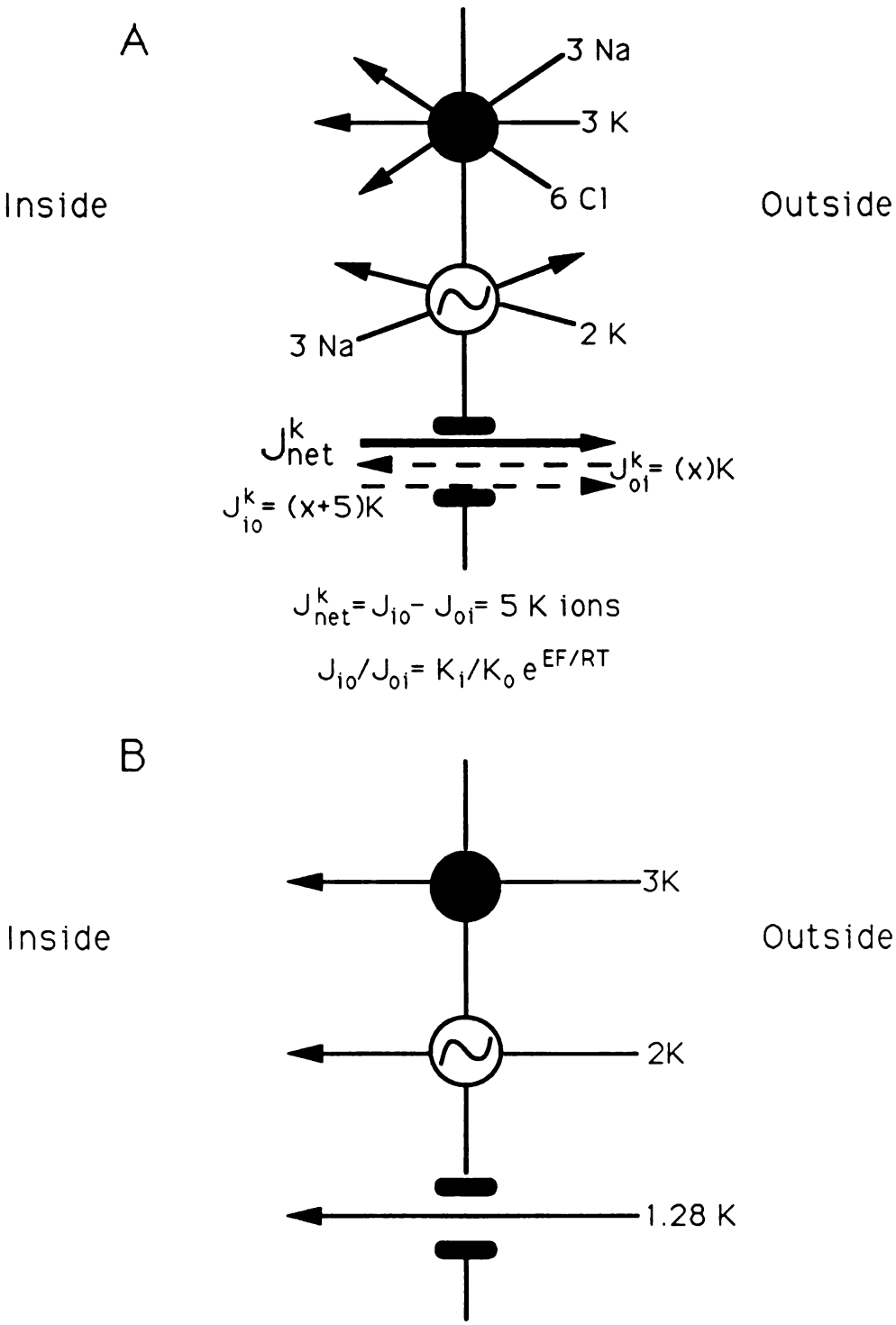

Figure 20. K movements across basolateral membrane. a) Calculation of influx and efflux through K conductance (see text for details). b) Relative K influxes through major transport pathways. The percentage of inhibition of ^{86}Rb uptake by bumetanide is predicted to be ~ 50%, by the analysis given in the text.

From the literature, the value of aK_i is 83 mM (Smith and Frizzell, 1984) and E is 38 mV (Widdicombe et al, 1985). In this study, aK_o is 4.05 mM. Using these figures, and the relationship noted earlier (J_{io}-J_{oi} = 5), the calculated values of J_{io} and J_{oi} are 6.28 and 1.28, respectively (Fig. 20a). Thus, the total basolateral influx of tracer ^{86}Rb of 6.28 is accounted for by 1.28 through the K conductance, 2 by the Na/K ATPase, and 3 via the Na/K/2Cl cotransporter (Fig. 20b). Maximal inhibition, then, of the cotansporter by serosal bumetanide would result in a 3/6.28, or ~47.8%, decrease in the total influx. This is not different from the ~50% inhibition by bumetanide found in the present studies. These data are therefore consistent with the model in which basolateral Cl uptake is by means of an Na/K/2Cl cotransporter.

The analysis offered in the present study supports a model in which the basolateral Cl uptake mechanism, in epithelial monolayers actively secreting Cl, is an Na/K/2Cl cotransporter. Experiments using isolated dispersed tracheal epithelial cells from dog, cow, and rabbit (Widdicombe et al, 1981; Musch and Field, 1989; and Liedtke et al, 1988) failed to show K-dependence and produced conflicting results regarding the regulation of the cotransporter. The observations concerning the K-dependence are in disagreement with indirect evidence from studies on the intact tissue (Welsh, 1984), which suggested that K was required. Thus, K dependence may be controlled by a host of factors—including osmotic stress and altered intracellular Ca^{++} levels—that may inadvertently and uncontrollably result from preparing dispersed cells. Thus, these variable observations should alert those who study Na-coupled Cl transport to the shortcomings of isolated dispersed cells.

The confluent cultured monolayer is an ideal system to study the cotransporter with either conventional I_{sc} measurements or isotope fluxes. These methods present many advantages over other techniques and preparations in charac-

terization of Na-coupled Cl transport. Single channel measurements have the following drawbacks. First, since the patch clamp technique assays for transport that generates current, electroneutral cotransport cannot be measured. Secondly, even if the carrier were electrogenic, the turnover rate of such a mechanism is far lower than that of a channel. Thus, any current produced would be below the level that conventional patch clamp amplifiers are able to measure. Finally, cultured epithelial cells grow in the same orientation as the native tissue, thus presenting problems in accessibility; even if basolateral Cl uptake were via a channel, measuring its activity would present significant difficulties. Isolated dispersed cells present problems associated with the enzymatic treatment integral to their preparation. These include the osmotic and chemical extremes that may modify native transport processes. Moreover, cell directionality is lost, rendering studies of vectorial transport difficult. Native tracheal epithelium has a serosal dead space of up to 1 mm, a feature that poses a significant limitation in studies requiring rapid basolateral exposure to tracers or drugs. On the other hand, confluent cultures of epithelial monolayers have a greatly reduced serosal dead space of ~10 μm, thus representing a major advantage over using the native tissue.

Thus, using two complementary approaches, the features of the basolateral Cl uptake mechanism in primary cultures of canine tracheal epithelial cells have been characterized. Electrical measurements support the model in which a basolaterally located transport pathway important in the vectorial transport of Cl is coupled tightly to a K conductance. Isotope flux measurements indicate that the presence of Na, and K is critical to the function of the Cl uptake pathway. Only in the presence of these ions does bumetanide inhibit transport. Bumetanide shows no inhibition of uptake in the absence of Na and Cl. These observations are evidence that the canine tracheal epithelium, when maintained in

primary culture, secretes Cl by a bumetanide-sensitive mechanism involving the interdependent basolateral uptake of Cl, Na, and K. This process may very well be the Na/K/2Cl cotransporter described for other tissue preparations. Together with previous work from this laboratory characterizing the apical membrane Cl conductance (Fong et al, 1988), the conclusions derived from the present study form a basis from which further work examining the interaction of apical and basolateral membranes during secretion can emerge.

Summary

The properties of the basolateral Cl uptake mechanism in primary cultures of dog tracheal epithelial cells were investigated. The cells were grown to confluency on collagen-coated polycarbonate membrane filters and used in either Ussing chamber or isotope flux experiments. Ba^{++} caused a dose-dependent inhibition of I_{sc}. An Eadie-Hofstee plot of these data revealed two (high and low affinity) binding sites for Ba^{++}, with maximal inhibition of 82.8 ± 0.0 %. This is not different from the 5/6 inhibition expected for Na/K/2Cl cotransport, but is significantly greater than the 2/3 inhibition expected for NaCl symport. Next, the effects of 10^{-4} M bumetanide on the basolateral uptake of ^{86}Rb were investigated. Bumetanide inhibited the initial rate of ^{86}Rb uptake by ~50 ± 10%. ^{86}Rb flux was decreased by the substitution of Cl by isethionate, as well as the replacement of Na by N-methyl-D-glucamine. Bumetanide had no effect on ^{86}Rb uptake in Na- or Cl-free media. There was no significant accumulation of ^{86}Rb when the tracer was introduced only on the apical side of the monolayer. The data support the presence of a basolateral Na/K/2Cl cotransporter.

Chapter Five

Conclusions

The studies undertaken in this dissertation research have led to observations that not only support, but also refine, a widely accepted model describing Cl secretion by airway epithelium. The major critical components determining Cl secretion—the basic transport mechanisms residing in the polarized membranes of epithelial cells, and their regulation—were recognized and studied individually. This task was facilitated by the application of powerful new techniques, as well as the strategic use of classical methods.

The conductive route for Cl exit across the apical membrane was demonstrated, in these studies, to possess the hallmarks of transporters categorized traditionally as channels. In addition to verifying, using a completely new technique, the basic conductive and Cl-selective properties of the pathway, insight into the molecular mechanism of Cl permeation was obtained. The conductance is saturable at high, but linear at very low, Cl concentrations. This observation is consistent with channel type mechanisms that do not obey the independence principle (Hodgkin and Huxley, 1952). In addition, the activation energy for Cl permeation, obtained from temperature dependence studies, indicates that Cl ions move as if in free solution. Thus, an aqueous pore mechanism is implicated.

Further studies tested for the involvement of two different systems in the regulation of the Cl channel. One of these, the cAMP-stimulated protein kinase pathway was demonstrated previously, by other groups using different techniques, to be involved in channel gating. The studies presented in this dissertation found no effect of the catalytic subunit of protein kinase A on Cl transport. The possibility that the conductance is directly gated by a G protein was suggested by the demonstration that the apical membranes are rich in an ADP-ribosylated, pertussis

toxin-sensitive protein. While the studies presented here do not present evidence for a role for direct channel gating by an apical membrane G protein, the possible involvement of a G protein endogenous to the basolateral membrane in regulation of the conductance is not ruled out. It will prove interesting to compare these data, and their implications, with those from any analogous future studies using different approaches and methods.

Finally, the work presented in this dissertation demonstrates that basolateral Cl uptake by tracheal epithelial cells, relevant to the process of transepithelial Cl secretion, proceeds via an Na/K/2Cl cotransporter. Using two independent approaches—consideration of the maximal inhibition of Cl secretion-associated I_{sc} by basolateral Ba^{++}, as well as the bumetanide and ion replacement sensitivity of ^{86}Rb uptake—the K dependence of the basolateral cotransporter was shown. With respect to the past—and often conflicting—literature, it must be emphasized that this observation does not rule out the presence of alternative routes of Na-dependent Cl uptake. Indeed, in cells that are subjected to various osmomechanical stresses, such mechanisms may prove the major component of bumetanide-sensitive Cl uptake.

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