J. Membrane Biol. 88, 149–163 (1985)

The Journal of
Membrane Biology

Ion Transport by Primary Cultures of Canine Tracheal Epithelium: Methodology, Morphology, and Electrophysiology

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Summary. Canine tracheal epithelial cells were isolated by enzymatic and mechanical dispersion and cultured on permeable supports. The cells formed confluent monolayers and retained most of the morphologic characteristics of the intact epithelium, including apical microvilli, apical tight junctions, and a moderately interdigitated lateral intercellular space. The cells also retained the functional properties of the epithelium. The monolayer responded to addition of isoproterenol with the characteristic changes in cellular electrical properties expected for stimulation of Cl secretion: isoproterenol increased transepithelial voltage, depolarized apical membrane voltage, and decreased both transepithelial resistance and the ratio of apical-to-basolateral membrane resistance. Examination of the cellular response to ion substitutions and inhibitors of Cl secretion indicate that the cultured monolayers retain the same cellular mechanisms of ion transport as the intact epithelium. Thus, primary cultures of tracheal epithelium may provide a useful preparation for future studies of the mechanism and regulation of Cl secretion by airway epithelia.

Key Words chloride · secretion · epithelium · trachea · cultured cells · electrophysiology · airways

Introduction

Cultured epithelial cells offer several advantages over the intact epithelium in the study of the mechanisms of ion transport. They can provide a more homogeneous cell population, and there is no thick submucosal connective tissue layer. Cultured cells can also be more readily studied with biochemical, optical, and electrophysiological (patch-clamp) techniques. Finally, by using cultured cells it may be possible to study human epithelia that are otherwise difficult to obtain.

In previous studies we have examined the mechanisms of Cl secretion by canine tracheal epithelium. The purpose of this study was to obtain primary cultures of canine tracheal epithelium and to compare the ion transport functions of the cultured cells with those of the intact epithelium. Such a comparison is essential if cultured cells are to be used to investigate the mechanisms and regulation of ion transport. We cultured cells on permeable supports using techniques similar to those described by Coleman et al. (1984). We have examined the cell morphology and the ion transport functions, especially the Cl-secretory process, using intracellular microelectrode techniques.

Symbols

ψ	electrical potential difference, in mV
R	electrical slope resistance in Ω cm^2
f_R	fractional resistance of the apical cell membrane, $\Delta\psi_a/\Delta\psi_t = R_a/(R_a + R_b)$
a, b, p, t	as subscripts refer to apical, basolateral, paracellular or transepithelial, respectively.

Materials and Methods

Cell Isolation

The methods of cell isolation are similar to those described by Coleman et al. (1984). Mongrel dogs (30 to 40 kg) of either sex were anesthetized with phenobarbital (25 mg/kg). The tracheal circulation was perfused under sterile surgical conditions to reduce the number of red blood cells in the final cell suspension. The tracheal circulation was isolated by ligation of both carotid arteries above the insertion of the superior thyroid artery and by ligation of the subclavian and proximal carotid arteries. Both common carotids were then cannulated and perfused with 2 liters of cold (5°C) Ringer's solution containing (in mM): NaCl, 118.9; K_2HPO_4, 2.4; KH_2PO_4, 0.6; $CaCl_2$, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 5; and HEPES buffer, 20. The solution also contained penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin (2.5 μg/ml). The first 100 ml of perfusion solution contained 60 units/ml heparin. The heart was stopped by injection of 3 M KCl. Blood and perfusate were removed by suction through a catheter inserted in the superior vena cava.

After the perfusion was complete, the trachea was transected just below the larynx and at the tracheal bifurcation and then removed. Under sterile conditions, the trachea was opened longitudinally and scored with a scalpel. Strips of epithelium

were then pulled away from the underlying submucosa and cartilage. The epithelial strips (approximately 100 mg wet weight) were minced with a scissors and placed in a flask with the enzyme solution. The enzyme solution contained the same materials as the perfusion solution except that it was nominally Ca and Mg free (EGTA was not added). In addition, the enzyme solution contained 1% bovine serum albumin (fraction V, Sigma), 0.02% collagenase (type I crude, Sigma), DNAse (100 μg/ml, Sigma), and dithiothreitol (5 mM, Sigma). The cells were incubated in 100 ml of enzyme solution (room temperature and ambient O_2 and CO_2) with intermittent (approximately 50% of the time) stirring with a magnetic stir bar. After 90 min, the supernatant was removed and filtered through gauze. Then, 100 ml of fresh enzyme solution was added and the tissues were incubated for another 90 min before the supernatant was again removed. A final collection lasted for 60 min. After each of the individual collections had been completed (90, 180 and 240 min) the cells were spun at 100 × g for 10 min, the supernatant was discarded, and the cells were resuspended in 5 ml of culture media and maintained at room temperature.

Cell Culture

When all three collections had been completed, the cells were pooled and "preplated" for 1 hr on uncoated tissue culture dishes (37°C, 5% CO_2) in order to remove fibroblasts which rapidly adhere to the dish. After preplating, the nonadherent cells were gently removed and counted with a hemocytometer. Cell viability was estimated by trypan blue exclusion. The average number of cells obtained from one animal was $1.5 \pm 0.2 \times 10^8$ (n = 13) with a viability of 93 ± 2%. The cells were then plated at a density of 2.5 to 5 × 10^5 cells/cm^2. The culture medium consisted of a 1-to-1 mixture of Dulbecco's Modified Eagles Medium containing 1 g/liter glucose and Ham's F-12 medium. The medium was supplemented with 5% fetal calf serum, nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin. The medium was changed 16 to 20 hr after plating and then 3 times a week. The medium was not changed for 20 hr before studying the electrical properties.

Cells were grown on polycarbonate filters (0.8 μm pore size, Nucleopore Corp., Calif.). The filters and the culture dish were coated with human placental collagen (type VI, C-7521, Sigma) at a final concentration of approximately 12 μg/cm^2. The collagen solution was always prepared fresh the day before harvesting the cells by dissolving the collagen at a ratio of 50 mg collagen in 100 ml water containing 0.2% glacial acetic acid. The solution was sterilized by filtration. The polycarbonate filter was incubated in the collagen mixture for 18 to 20 hr, then the collagen solution was removed and the filter was rinsed with phosphate-buffered saline. The filter was then air-dried for 4 to 6 hr at room temperature. The polycarbonate filters were attached to the bottom of the petri dishes in three or four spots by touching the filter with a hot soldering iron. Thus, the culture media had access to both surfaces of the monolayer. The culture media and supplements were obtained from the Cancer Research Facility (University of Iowa).

Before settling on the culture conditions listed above, we tried a variety of other media supplements as well as a variety of different substrate conditions. The media supplements that we examined were: epidermal growth factor (10 ng/ml, Sigma), transferrin (5 μg/ml, Sigma), 3-3-5-triiodo-DL thyronine (5 × 10^{-12} M, Sigma), hydrocortisone (0.1 μg/ml, Sigma), theophylline (10^{-6} to 10^{-4} M, Sigma), prostaglandin E_1 (10^{-9} to 10^{-7} M, The Upjohn Co., Mich.), retinoic acid (10^{-8} to 10^{-6} M, Sigma), selenous acid (5 μg/ml, Collaborative Research Inc., Mass.), 2-aminoethanol (10^{-6} M, Sigma), DMSO (10^{-5} M, Fisher Scientific, Inc., N.J.), and varying concentrations of fetal calf serum (0.4 to 10%). The other substrates that we tried were: calf-skin collagen (Cooper Biomedical, Pa.), rat-tail collagen, and poly-L-lysine hydrobromide (0.1% solution, Sigma). However, we saw no appreciable improvement in either cell attachment to the substrate or growth rate with any of these various interventions.

Morphologic Studies

We examined living cells in culture with an inverted microscope (IM-35, Carl Zeiss), using either phase-contrast or differential-interference contrast optics. For electron microscopy, monolayers were fixed in fresh 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.4). Cells were prepared for transmission, scanning, and freeze-fracture electron microscopy by the University of Iowa central electron microscopy facility using standard techniques.

Electrophysiologic Techniques

Examination of the transepithelial and transmembrane electrical properties were performed using techniques previously described for the intact epithelium (Welsh et al., 1982). Cell monolayers grown on filters were mounted in a micropuncture chamber (0.125 cm^2) that allowed measurement of transepithelial voltage and resistance. Resistance of the fluid and collagen-coated polycarbonate filter (approximately 15 to 20 Ω cm^2) were subtracted from all measurements. Cells were impaled using conventional microelectrodes (filled with 0.5 M KCl) with a resistance of 100 to 150 MΩ (when measured in 0.5 M KCl). The criteria for successful cellular impalements were the same as those we have previously used in intact tracheal epithelium (Welsh et al., 1982): 1) an abrupt negative deflection of the electrical potential sensed by the microelectrode tip; 2) a stable plateau value of the electrical potential difference whose magnitude was at least as large as the initial deflection obtained on impalement of the monolayer; 3) a stable (±2 mV) plateau value lasting at least 30 sec; and 4) an abrupt return to the baseline (preimpalement) value (±2 mV) upon withdrawal of the electrode from the cell. During unilateral ion substitutions, no correction was made for liquid junction potentials because we used flowing KCl bridges directly connected to the calomel cells (in the efflux port of the chamber) to directly measure ψ_t.

For the functional studies, the cells were continuously perfused with a Ringer's solution containing (in mM): Na, 139.3; K, 5.4; Ca, 1.2; Mg, 1.2; Cl, 123.7; HPO_4, 2.4; H_2PO_4, 0.6; HCO_3, 20.5; and glucose, 10. The solution was bubbled with 95% O_2–5% CO_2 (pH 7.4 at 37°C). For ion substitution studies, gluconate was substituted for Cl and tetramethylammonium (TMA) was substituted for Na.

Amiloride was a generous gift of Merck, Sharp and Dohme Research Laboratories, Westpoint, Pa. Bumetanide was a generous gift of Hoffman-LaRoche Inc., Nutley, N.J. Other chemicals used were isoproterenol (Elkins-Sinn, Inc., Cherry Hill, N.J.) and ouabain, indomethacin, and barium chloride (all from Sigma).

Sodium and K concentrations were measured with a flame photometer (Instrumentation Laboratory, Inc., Mass.). Chloride

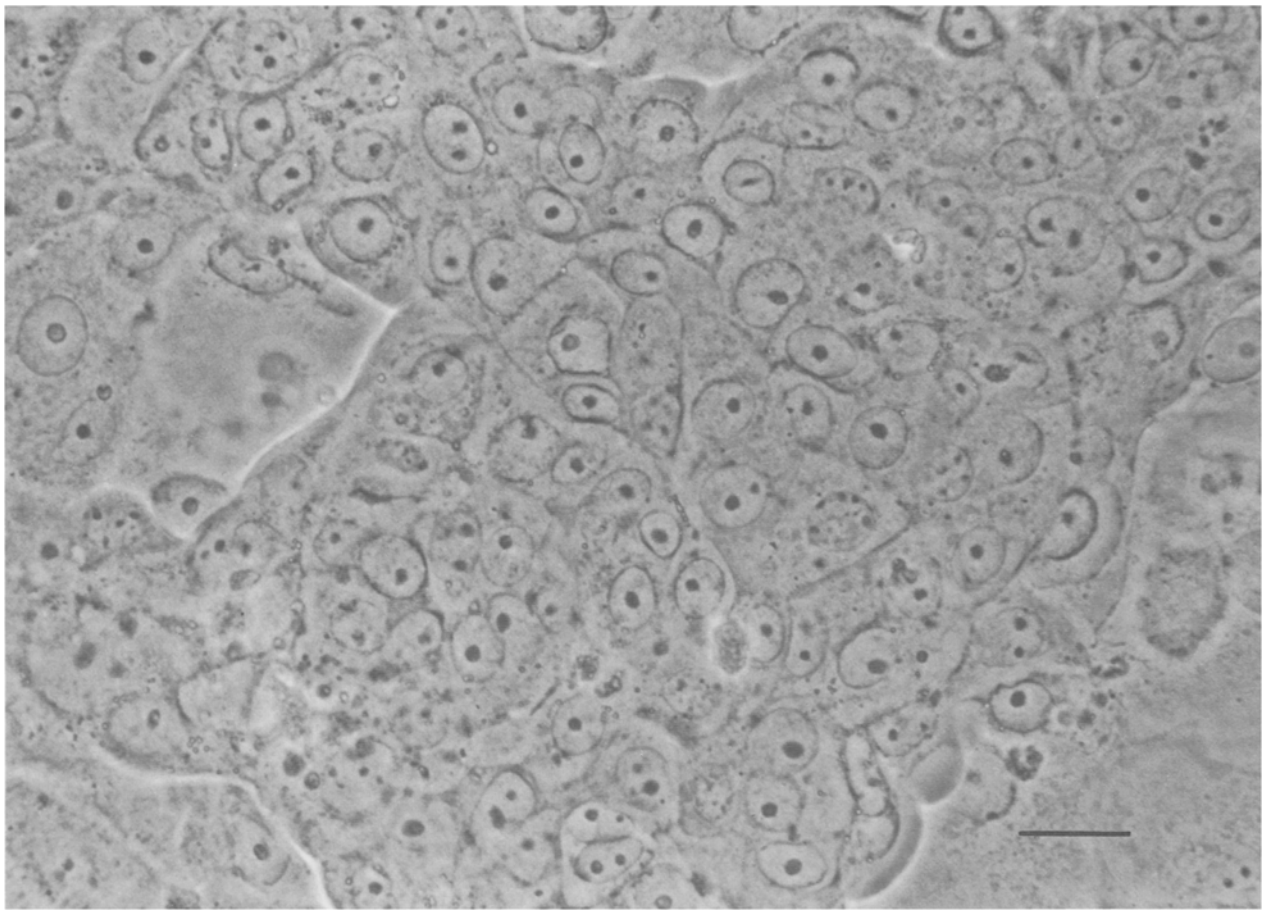


Fig. 1. Phase-contrast photomicrograph of cultured tracheal epithelial cells grown on a collagen-coated plastic vessel. Bar = 20 μm

was measured with a Cotlove chloridometer (Laboratory Glass and Instruments Corp., N.Y.).

Results

Morphology

It was not possible to directly observe cells growing on the permeable supports, because the filters are opaque. Therefore, we also seeded cells on collagen-coated plastic culture dishes and glass coverslips. Figure 1 shows a phase-contrast photomicrograph taken three days after the cells were plated at a density of 5×10^4 cells/cm^2. The figure shows a small group of cells that have grown to confluency (although the entire culture was not yet confluent); the cells have a polygonal "cobblestone" shape, a characteristic similar to that observed with other cultured epithelial and endothelial cells. Ciliated cells were occasionally observed scattered throughout the monolayer.

Figure 2 shows a scanning electron photomicrograph of the surface of cells cultured on polycarbonate filters. The most prominent feature is the microvilli that cover the apical membrane. Microvilli also cover the surface of intact tracheal and other airway epithelia (Rhodin, 1966; Frasca et al., 1968; Yoneda, 1976; Marin et al., 1979; and *personal observations*).

Figure 3 is a transmission electron photomicrograph of cells grown on a polycarbonate filter. There are several features to note. First, the microvilli that were demonstrated in the scanning electron photomicrographs are located only on the apical surface. Second, the area of the junctional complex, including the tight junction and desmosomes, is apparent just beneath the apical surface (arrow). Third, it is apparent that there is some interdigitation of the lateral intercellular space (arrowheads), just as has been observed in intact tra-

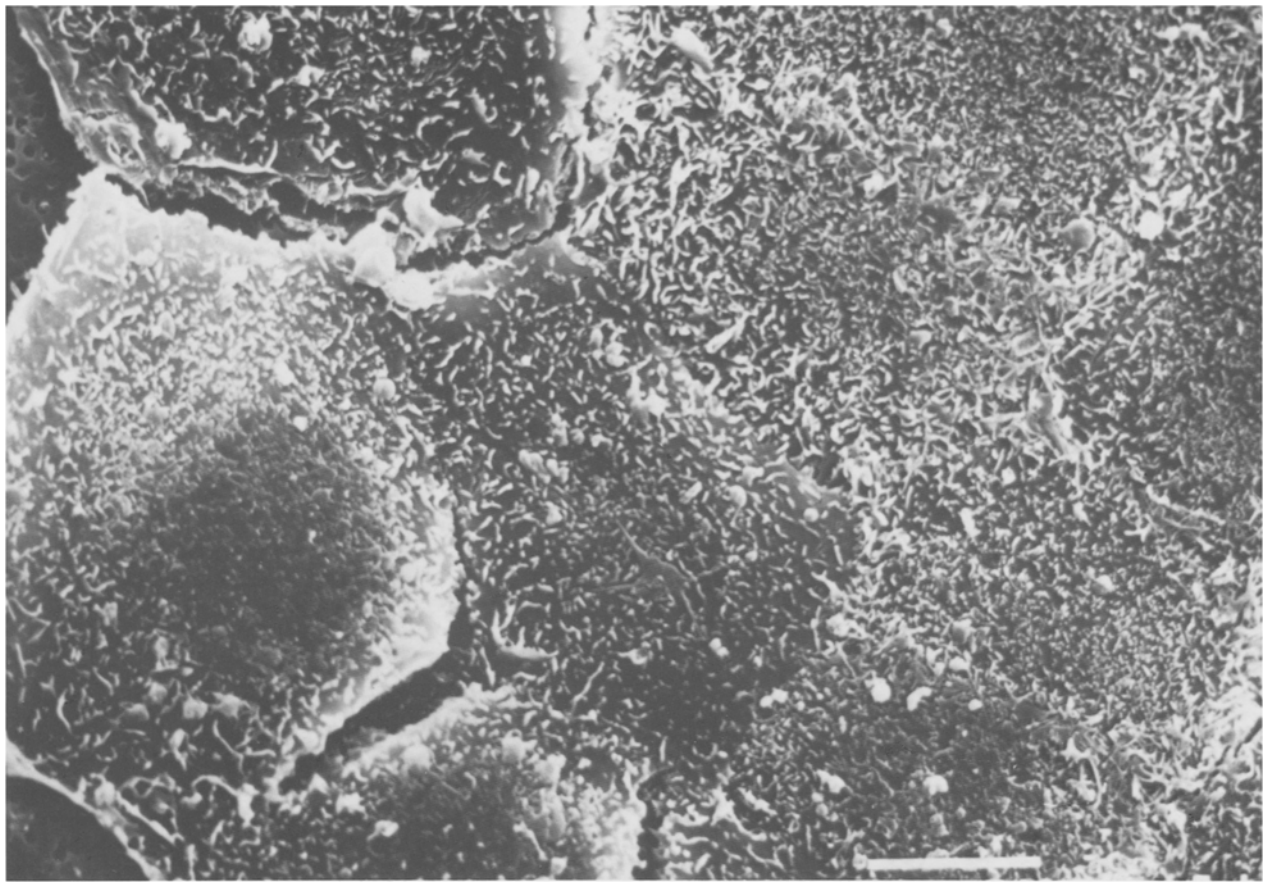


Fig. 2. Scanning electron photomicrograph of cells grown on a polycarbonate filter for 4 days. A portion of the polycarbonate filter can be seen in the upper left corner. Bar = 10 μm

cheal epithelium. Finally, the cytoplasm has an organization similar to that of the intact epithelium with apical mitochondria and a prominent endoplasmic reticulum (Rhodin, 1966; Frasca et al., 1968; Yoneda, 1976; Marin et al., 1979; and *personal observations*). No globet or mucus cells were observed.

To better demonstrate the presence of intact tight junctions we used freeze-fracture techniques to examine the epithelium; a representative photomicrograph is shown in Fig. 4. A linear pattern of membrane particles characteristic of epithelial tight junctions separates the apical and basolateral membrane surfaces (arrowheads). The apical membrane surface shows evidence of multiple microvilli (small arrows) which have been fractured from the specimen. The basolateral membrane had a higher concentration of intramembrane particles and contained structures consistent with gap junctions (large arrow).

Dome Formation

After day 4 in culture, we often saw "domes" in monolayers grown on plastic or glass. Domes represent confluent sheets of cells that have lifted up from the culture dish but remain attached at the edge of the dome (Tanner et al., 1983; Sugahara et al., 1984). Dome formation results from active salt and water transport from the apical to the basolateral surface of the cells. Subsequently, the cell layer is pushed up away from the impermeable culture dish in areas that are not as firmly adherent to the substrate. Figure 5 shows a representative example.

The domes varied in size and shape. Most appeared to have a circular base, but occasionally the X and Y measurements differed by up to 30%. The diameter of domes was measured with a calibrated eyepiece reticle and the height was determined from the change in objective height required to bring the

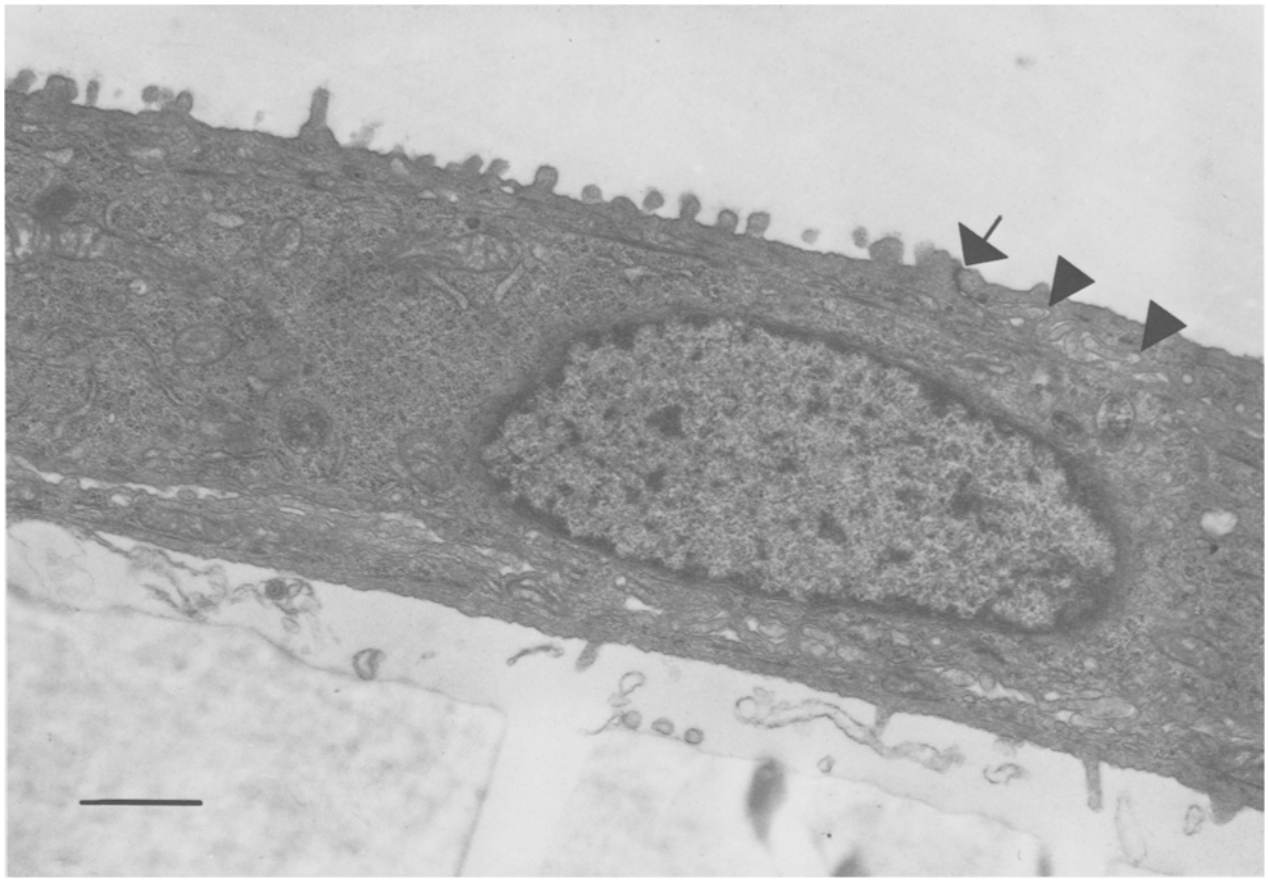


Fig. 3. Transmission electron photomicrograph of cells grown on polycarbonate filter for 6 days. The filter is visible beneath the monolayer. Small arrow: junctional complex. Arrow heads: lateral intercellular space. Bar = 2 μm

base and top of the dome into focus. The domes frequently reached a mean diameter of 300 μm and occasionally, 500 μm. The height of the dome above the culture dish was often 100 μm and was occasionally as great as 140 μm. The calculated volume of fluid in the domes based on measured height, diameter, and the assumption that domes are sections of spheres (Tanner et al., 1983), ranged up to 10 nanoliters.

Electrical Properties

To examine the electrical properties, monolayers, grown on permeable supports, were mounted in the micropuncture chamber. Figure 6 *a–c* shows the relationship between monolayer age and transepithelial and intracellular electrical properties. Transepithelial resistance increased progressively following plating, reaching a peak at day 9 or 10, and then tended to decline (Fig. 6*a*). The spontaneous transepithelial electrical potential difference was usually small, ranging from zero to 1 mV, with occasional values of 1 to 3 mV before addition of secretagogue. The time course of increase in values of ψ_t paralleled the changes in R_t (Fig. 6*a*). We saw no consistent pattern of change in short-circuit current I_{sc}.

The electrical potential difference across the apical membrane (ψ_a) tended to decrease as the age of the monolayer increased. Figure 6*b* shows that ψ_a was most negative initially, and then depolarized slightly with time. This pattern was not explained by consistent time-dependent changes in the fractional resistance of the apical cell membrane (f_R) (Fig. 6*c*), which remained fairly stable until 10 days in culture. After 10 days the value of f_R decreased. For the remainder of the studies, cells were used between 5 and 10 days after plating.

Although there were large variations in ψ_a depending upon the specific monolayer and time in

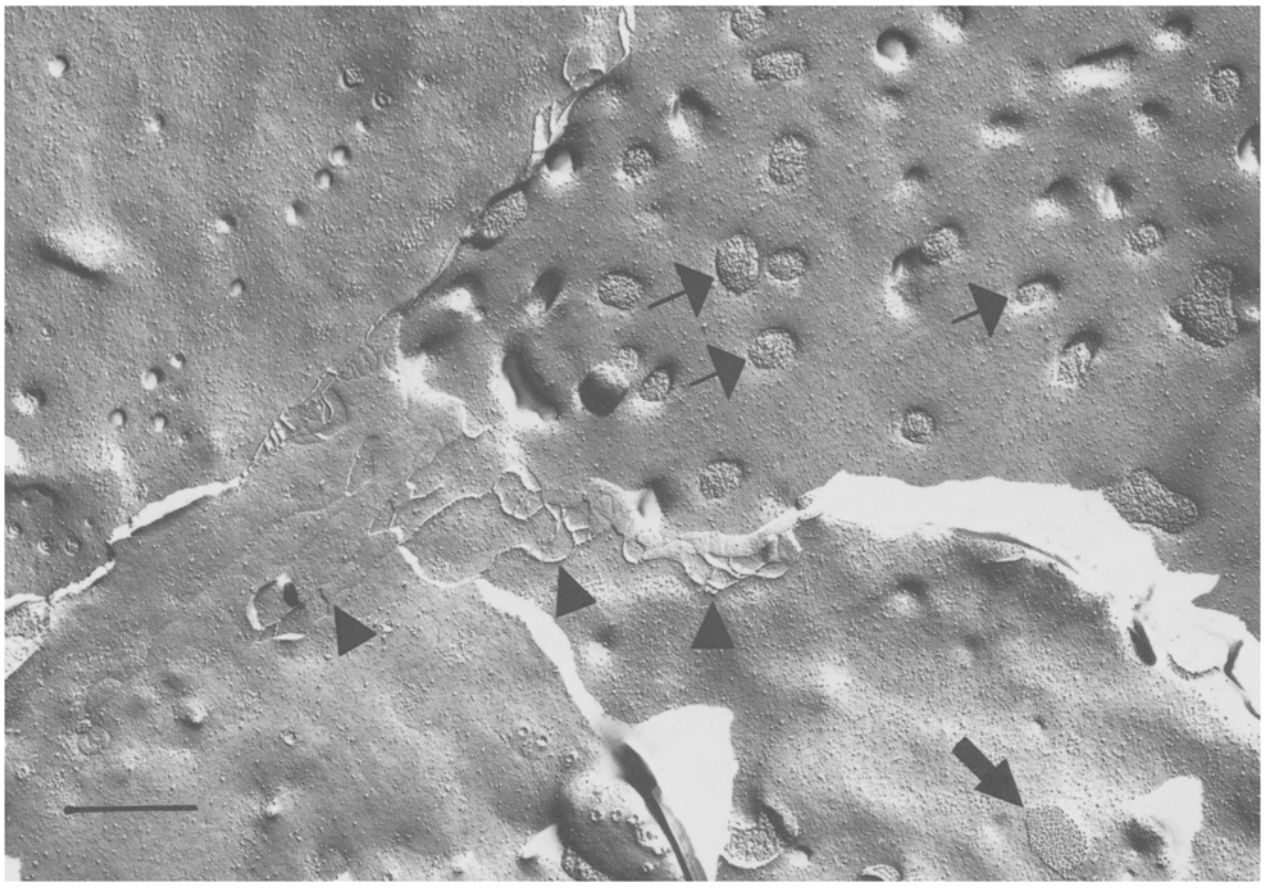


Fig. 4. Freeze-fracture electron photomicrograph of tracheal epithelium grown on polycarbonate filter for 6 days. Small arrows: apical microvilli. Large arrow: gap junction. Arrow heads: tight junction. The tight junction shows portions of both the E (left) and P (right) face of the fracture. Bar = 0.5 μm

culture, variation of ψ_a in a given monolayer was much less. Figure 7 shows values of ψ_a obtained in five individual monolayers, each obtained from a different animal. In each of these five monolayers, labeled *A–E*, at least four separate cellular impalements were made. The figure shows that the range of ψ_a in a given monolayer was small.

The formation of domes by cells grown on plastic suggested that the cells might contain an active Na absorptive process. Therefore, we added amiloride (10^{-4} M) to the mucosal surface of monolayers grown on permeable supports; Fig. 8 shows one of the more pronounced examples of the response to amiloride. In this monolayer amiloride abruptly decreased ψ_t, hyperpolarized ψ_a, and increased R_t and f_R. These changes indicate the inhibition of an apical membrane Na conductance resulting in a decrease in transepithelial Na absorption (Welsh et al., 1983). However, amiloride did not always have an effect; most frequently no electrical response was seen after adding amiloride.

In intact tracheal epithelium endogenous prostaglandin production may underlie the spontaneous Cl secretion (Al-Bazzaz et al., 1981; Smith et al., 1982). However, when we added indomethacin (10^{-6} M, mucosal solution) we saw no measurable effect on the electrical properties of the monolayers.

To determine if monolayers retained the ability to secrete Cl we added isoproterenol 5×10^{-6} M to the submucosal bathing solution. Figure 9 shows the typical response: ψ_t increased and ψ_a depolarized. In addition both R_t and f_R decreased (as indicated by the decreased voltage deflections in ψ_t and ψ_a produced by constant transepithelial current pulses). These changes are qualitatively similar to those observed in intact tracheal epithelium (Welsh et al., 1982, 1983; Shorofsky et al., 1983), as well as other Cl-secreting epithelia (Nagel & Reinach, 1980; Greger & Schlatter, 1984). This pattern suggests a secretagogue-induced increase in the apical membrane Cl permeability that leads to a decrease

Table 1. Effect of submucosal isoproterenol (5×10^{-6} M) on steady-state cellular electrical properties[a]

Condition	ψ_t (mV)	ψ_a (mV)	R_t (Ω cm^2)	f_R	I_{sc} (μA cm^{-2})
Baseline	0.8	−40	270	0.73	4
	±0.4	± 5	±65	±0.05	±2
Isopro-	1.7	−25	214	0.36	11
terenol	±0.5	± 3	±49	±0.07	±3
Δ	0.9	15	−56	−0.37	7
	±0.2	± 3	±18	±0.08	±2
P	0.0026	0.0005	0.013	0.0013	0.0069

[a] Δ represents the difference between the two periods and *P* is calculated from the paired *t*-test. Values are means ± SEM and were obtained during a single cellular impalement in each of 10 monolayers. I_{sc} was calculated from ψ_t and R_t for each monolayer.

in R_a (and secondarily, a decrease in R_t), and an increase in ψ_t resulting from a depolarization of ψ_a, as ψ_a approaches the electromotive force for Cl across the apical membrane. (This proposed mechanism for the depolarization of ψ_a depends upon the assumption that the intracellular Cl concentration is less than the extracellular Cl concentration, as it is in the intact epithelium (Welsh, 1983*d*; Shorofsky et al., 1984).)

The changes in transepithelial and transmembrane electrical properties shown in Fig. 9 were representative of our findings. Table 1 shows the mean electrical properties for a group of 10 monolayers (from six different culture groups) in which a single impalement was maintained during the baseline period and following addition of isoproterenol. The table also shows the I_{sc} calculated from ψ_t and R_t. The mean results are also consistent with the notion that isoproterenol stimulates secretion.

In the intact tracheal epithelium, stimulation of Cl secretion leads to both a decrease in apical resistance and a decrease in basolateral resistance (which results from an increase in basolateral K conductance) (Welsh, 1983*b*; Smith & Frizzell, 1984). The coupling of apical and basolateral conductances, so that they parallel one another when the rate of transepithelial transport changes, is an important regulatory process that is required for cell homeostasis in all epithelia (Schultz, 1981; Diamond, 1982). Examination of Fig. 9 shows that the monolayers do not display the dramatic biphasic changes in ψ_a and f_R that we have previously observed when epinephrine is added to the intact epithelium (Fig. 7, Welsh et al., 1982; Fig. 3, Welsh et al., 1983). Rather, the pattern of change is similar to that which we observed with addition of prostaglandin E_1 (Fig. 9, Welsh et al., 1982). Figure 10 shows the time course of change in cell electrical properties obtained following addition of isoproterenol. The results represent the mean values obtained from six monolayers. Examination of Fig. 10 shows that ψ_t continues to increase and R_t continues to decrease for 120 sec following the onset (time zero) of the electrical response. However, the changes in ψ_a and f_R were slightly dissociated (in time) from those of ψ_t and R_t; ψ_a and f_R reach minimum values, and then increase slightly. Although these changes are subtle, they were consistent findings. They suggest that, following addition of isoproterenol, both apical and basolateral resistances fall, but the fall in basolateral resistance lags slightly behind the fall in apical resistance.[1]

Cellular Mechanism of Ion Transport

Previous studies in intact canine tracheal epithelium have led to the development of a model of Cl secretion (Welsh et al., 1982) which may apply to a variety of secretory epithelia (Frizzell et al., 1979). In this model the Na-K-ATPase is located at the basolateral membrane and provides the energy for both Na absorption and Cl secretion by maintaining a low intracellular Na concentration and a high K concentration. Cl secretion is a two-step process: Cl is accumulated across the basolateral membrane by coupling to the movement of Na (which provides the electrochemical driving force) and probably K. Chloride exit occurs passively across a Cl-permeable apical cell membrane. Sodium absorption occurs via a passive conductive entry step at the apical membrane followed by an active exit step at the basolateral membrane via the Na-pump. Potassium that enters in exchange for Na on the Na-K-ATPase is recycled across the K-conductive basolateral membrane. To determine if cultured tracheal epithelium has a similar mechanism of transport, we used intracellular electrophysiologic techniques to examine the effect of ion substitutions and agents known to inhibit ion transport.

[1] Further evidence to suggest that the decrease in R_a is accompanied by a decrease in R_b can be obtained from consideration of an equivalent electrical circuit model of the epithelium (Welsh et al., 1983). If one assumes that isoproterenol only decreases R_a, and not R_b or R_p, values for each of the resistances can be calculated based on values of R_t and f_R obtained before and after addition of isoproterenol (Welsh et al., 1983). When this calculation was made for each of the individual monolayers that contributed to Fig. 10, a negative value of resistance was obtained. This indicates that the assumption that isoproterenol only affects R_a is wrong. When the results of these calculations are taken together with the changes shown in Fig. 10, the results are most consistent with the notion that isoproterenol decreases both R_a and R_b.

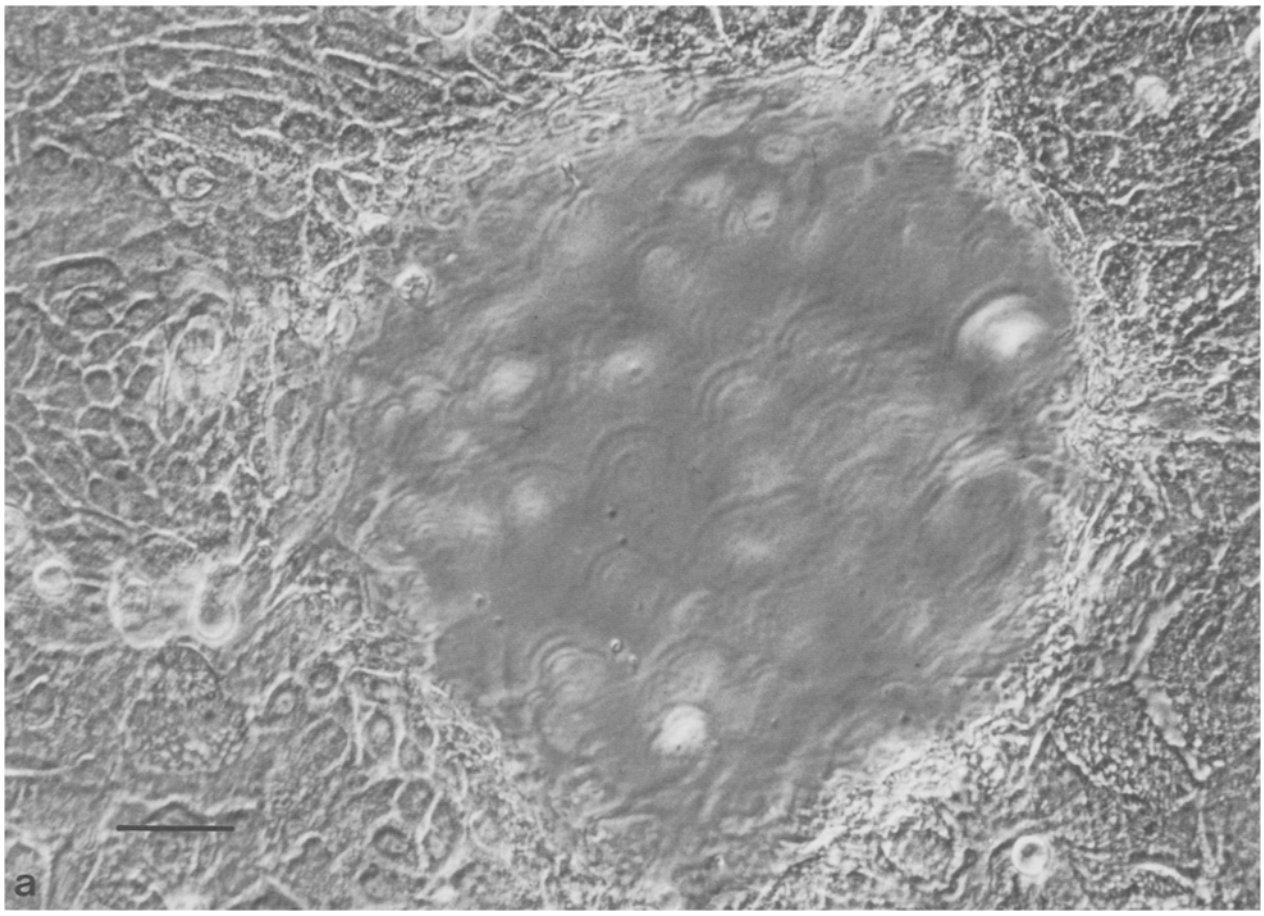
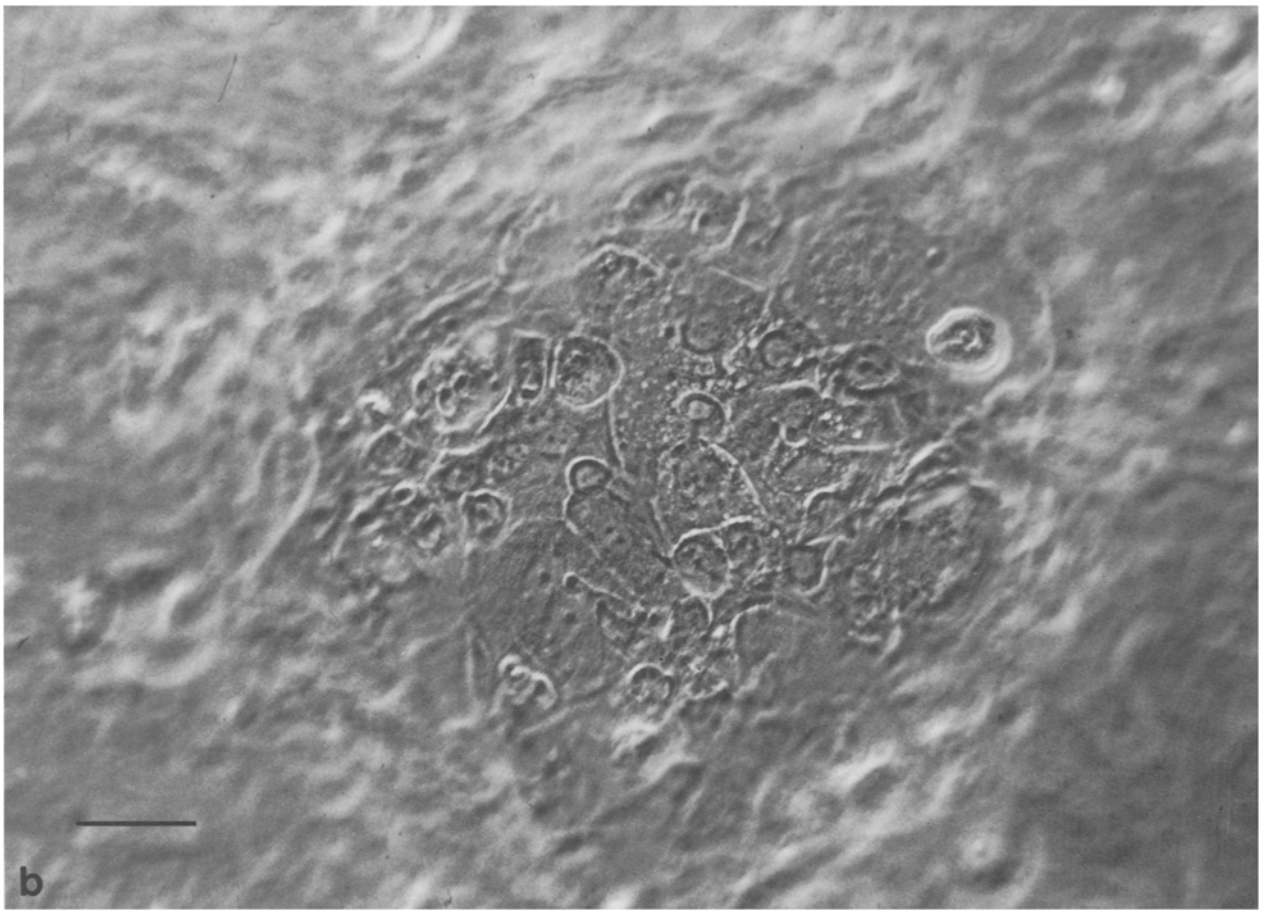


Fig. 5. Phase-contrast photomicrograph showing dome formation in cells grown on collagen-coated plastic petri dish for 5 days. *a* and *b* show the same field. *a* was taken at the level of the cells attached to the petri dish. *b* (*facing page*) was taken with the cells at the top of dome in focus, 53 μm above the level of *a*. Bar = 50 μm

Table 2. Effect of Cl substitutions on cellular electrical properties[a]

Condition (n)	[Cl] m − s	ψ_t (mV)	ψ_a (mV)	ψ_b (mV)	R_t (Ω cm^2)	f_R
Baseline	124 − 124	+ 1.3 ± 0.8	−39 ± 4	+41 ± 5	254 ± 63	0.75 ± 0.02
(7)	1 − 124	+10.7 ± 0.8*	−21 ± 4*	+32 ± 4	349 ± 104	0.74 ± 0.05
	124 − 1	− 8.8 ± 0.8*	−52 ± 5*	+43 ± 5	299 ± 77*	0.70 ± 0.03
	1 − 1	+ 0.4 ± 1.9*	−38 ± 4	+40 ± 5	405 ± 113*	0.77 ± 0.03
Isoproterenol	124 − 124	+ 1.2 ± 0.8	−18 ± 2	+19 ± 3	162 ± 53	0.34 ± 0.10
(5×10^{-6} M)	1 − 124	+14.5 ± 1.7*	+ 9 ± 2*	+24 ± 3	271 ± 103*	0.64 ± 0.09*
(6)	124 − 1	− 8.5 ± 1.3*	−44 ± 5*	+35 ± 6*	223 ± 70*	0.31 ± 0.10
	1 − 1	− 1.6 ± 1.3	−27 ± 3	+26 ± 4*	327 ± 109*	0.64 ± 0.07*

[a] The bathing solution Cl concentration [Cl] was decreased either unilaterally or bilaterally by substituting gluconate; m refers to the mucosal solution and s to the submucosal solution. ψ_b was calculated as $\psi_b = \psi_t - \psi_a$. (n) refers to the number of monolayers in each group. Values in each group (Baseline or Isoproterenol) were obtained from a single cellular impalement during which the ion substitutions were made. Values are means ± SEM. *$P < 0.05$ by paired t-test as compared to the value in the presence of symmetrical 124 mM [Cl].



First, we examined the effect of decreasing the Cl concentration of the bathing solution from 124 to 1 mM by substituting gluconate for Cl; Table 2 shows the results. Tissues were studied under both baseline conditions, and following addition of the secretagogue, isoproterenol (5×10^{-6} M). We tried to perform all the ion substitutions, before and after addition of secretagogues, in each tissue; however we were not always successful. Therefore, in Tables 2 through 4 there are different numbers of tissues studied in each group. Replacement of Cl in the mucosal, submucosal, or both solutions was performed in random order. The baseline and ion-substituted values for the electrical measurements always represent values taken during a single impalement, not a group of impalements under each condition. We usually were able to measure a "recovery" value after return to the normal Ringer's solution, to ensure that there were no artifactual changes during the course of the experimental intervention.

In unstimulated tissues, unilateral decreases in Cl concentration, [Cl], produced symmetrical changes in ψ_t and increased R_t. When [Cl] was reduced bilaterally, ψ_t decreased slightly as compared to control values and there was a further increase in R_t. These transepithelial changes suggest symmetrical Cl-conductive properties, a finding which would be best explained by a Cl-conductive paracellular pathway. This conclusion is suported by the intracellular measurements: neither unilateral nor bilateral Cl substitutions altered f_R, indicating the lack of a sizeable Cl conductance at either apical or basolateral membranes under nonstimulated conditions. The direction of the changes in ψ_a can be interpreted as being secondary to a Cl-conductive paracellular pathway and circular current flow.

Following stimulation of secretion with isoproterenol, ψ_a, ψ_b, f_R, and R_t decreased.[2] In stimulated tissues, unilateral Cl substitutions changed ψ_t

[2] Although the mean steady-state value of ψ_t was not statistically significantly altered by isoproterenol in the group of tissues shown in Table 2, this was the exception, rather than the rule, as indicated by the increase in ψ_t for the groups represented in Tables 1, 3 and 4. However, the combination of an unchanged ψ_t and decreased R_t in Table 2 suggests an increased rate of Cl secretion, i.e., the calculated short-circuit current increased.

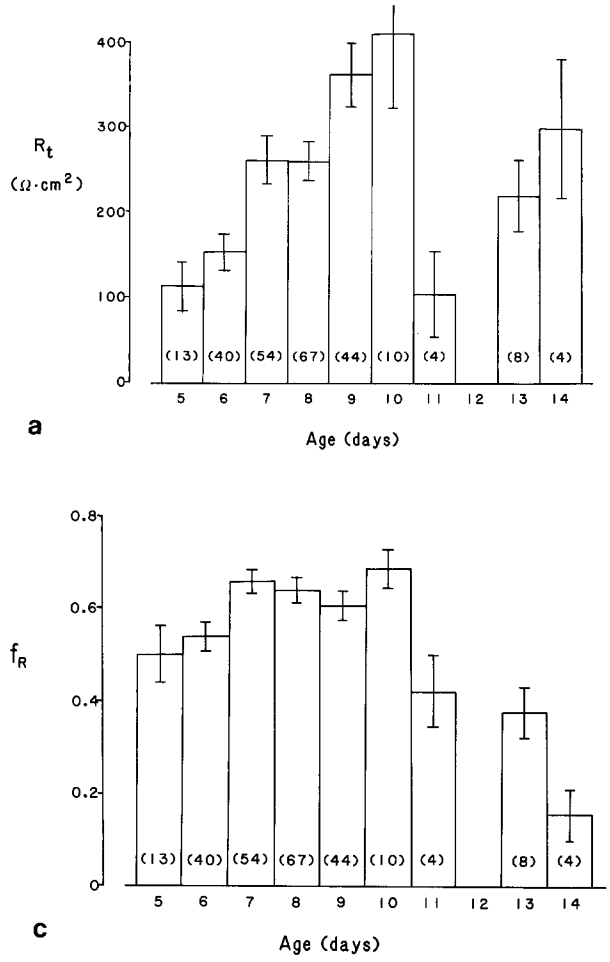


Fig. 6. Relation between age of monolayer and electrical properties. (*a*) Age *vs.* transepithelial electrical resistance R_t. (*b*) Age *vs.* electrical potential difference across the apical membrane ψ_a. (*c*) Age *vs.* fractional resistance of the apical cell membrane f_R. Numbers in parentheses indicate number of monolayers studied. When more than one cellular impalement was obtained in a single monolayer, the mean value for the monolayer was used

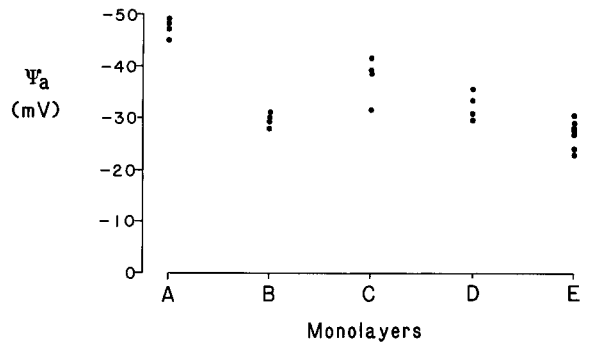


Fig. 7. Variability of apical voltage ψ_a within individual monolayers. Each monolayer ($A - E$) was obtained from a different animal. Each data point represents the value of ψ_a obtained from a single cellular impalement

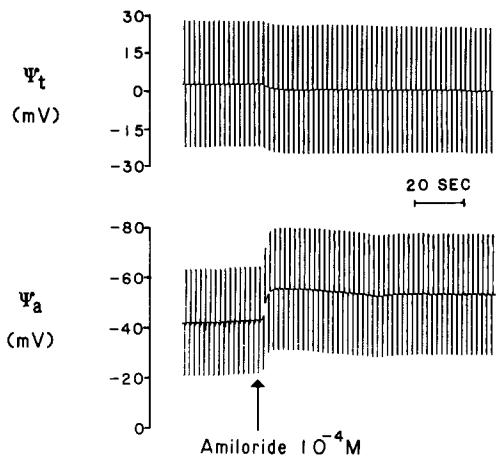


Fig. 8. Effect of mucosal amiloride (10^{-4} M) on cellular and transepithelial electrical properties. Voltage deflections resulted from bipolar square-wave, constant-current pulses (±80 μA cm^{-2})

and R_t in a manner similar to that observed in non-stimulated tissues, although the hyperpolarization of ψ_t and increase in R_t were larger with mucosal than submucosal substitutions. In addition, f_R increased significantly when mucosal [Cl] decreased either alone or in combination with a decrease in submucosal [Cl]. ψ_a also hyperpolarized when the [Cl] was decreased bilaterally. This pattern of change in ψ_a in response to unilateral decreases in

Table 3. Effect of K substitutions on cellular electrical properties (*See* legend of Table 2)

Condition (*n*)	[K] *m* − *s*	ψ_t (mV)	ψ_a (mV)	ψ_b (mV)	R_t (Ω cm^2)	f_R
Baseline	5.2 − 5.2	+0.3 ± 0.9	−39 ± 5	+39 ± 6	211 ± 45	0.68 ± 0.06
(5)	51 − 5.2	+1.6 ± 0.9*	−37 ± 5	+39 ± 6	190 ± 40*	0.68 ± 0.07
	5.2 − 51	−0.1 ± 0.8	−22 ± 2*	+22 ± 2*	194 ± 41*	0.84 ± 0.04*
	51 − 51	−0.7 ± 0.8*	−19 ± 2*	+19 ± 2*	184 ± 40*	0.83 ± 0.04*
Isoproterenol	5.2 − 5.2	+1.6 ± 0.9	−21 ± 3	+23 ± 4	121 ± 26	0.21 ± 0.05
(5×10^{-6} M)	51 − 5.2	+2.0 ± 1.0	−21 ± 4	+23 ± 4	111 ± 25*	0.21 ± 0.05
(6)	5.2 − 51	+0.7 ± 0.7*	−19 ± 3*	+19 ± 2*	114 ± 26*	0.33 ± 0.09*
	51 − 51	+0.8 ± 0.7*	−19 ± 2*	+19 ± 3*	106 ± 24*	0.35 ± 0.08*

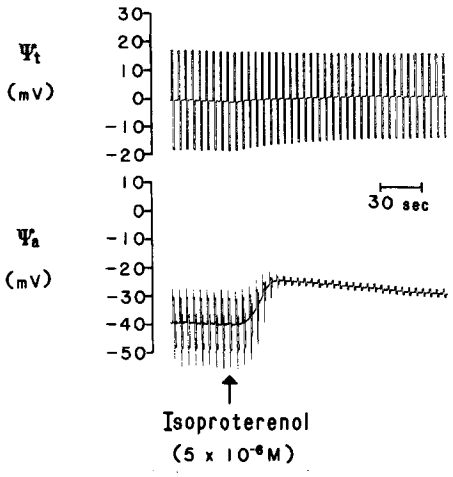


Fig. 9. Effect of submucosal isoproterenol (5×10^{-6} M) on cellular and transepithelial electrical properties. Voltage deflections resulted from bipolar square-wave, constant-current pulses (±80 μA cm^{-2})

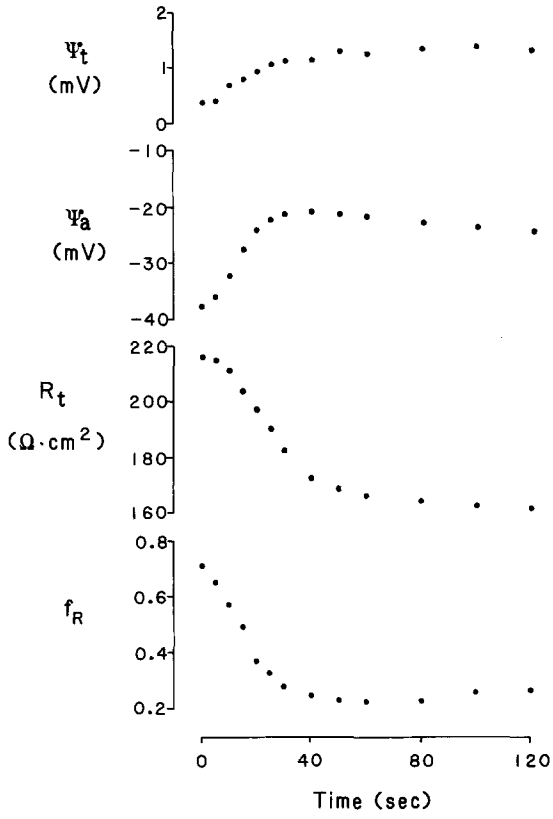


Fig. 10. Time course of the electrical response to submucosal isoproterenol (5×10^{-6} M). Values represent the mean obtained from studies in six monolayers. Time zero represents the onset of the response to isoproterenol

[Cl] is the same as that previously reported for intact tracheal epithelium (Welsh et al., 1982; Shorofsky et al., 1983). These results indicate that, following addition of isoproterenol, the apical cell membrane is Cl conductive.

Next, we examined the effect of K substitutions; K was increased from 5.2 to 51 mM in exchange for Na. The results, shown in Table 3, indicate that the basolateral membrane is K conductive under both stimulated and nonstimulated conditions. The most direct evidence for this conclusion is the observation that f_R increased and ψ_b depolarized when submucosal (or the combination of submucosal and mucosal) [K] increased. In contrast, an increase in mucosal [K] did not alter either f_R or ψ_b. The changes in ψ_a and f_R are smaller in isoproterenol-treated tissues, as would be expected if a Cl conductance dominates the cellular conductances. Unilateral elevation of the [K] also tended to decrease R_t and altered ψ_t in a manner expected if the paracellular pathway were slightly more conductive for K than for Na.

Finally, Table 4 shows the effect of decreasing the [Na] from 137 to 24 mM by replacement with TMA. In nonsecreting monolayers, there were small changes in ψ_t during unilateral substitutions, consistent with a transepithelial diffusion voltage for Na. In addition, there was a small hyperpolarization of ψ_a when mucosal [Na] decreased; this change would be consistent with a small Na conductance in the apical membrane. However, these changes were small, and because there were no significant

Table 4. Effect of Na substitutions on cellular electrical properties (*See* legend of Table 2)

Condition (n)	[Na] $m - s$	ψ_t (mV)	ψ_a (mV)	ψ_b (mV)	R_t (Ω cm^2)	f_R
Baseline	137 − 137	+0.7 ± 0.8	−34 ± 6	+34 ± 6	295 ± 91	0.74 ± 0.06
(5)	24 − 137	−1.1 ± 0.9	−37 ± 6*	+36 ± 7	300 ± 96	0.77 ± 0.07
	137 − 24	+2.2 ± 0.9*	−33 ± 5	+35 ± 6	285 ± 83	0.73 ± 0.05
	24 − 24	+0.4 ± 0.6	−35 ± 5	+36 ± 6	294 ± 86	0.79 ± 0.08
Isoproterenol	137 − 137	+1.6 ± 0.4	−17 ± 2	+19 ± 2	90 ± 27	0.26 ± 0.07
(5×10^{-6} M)	24 − 137	−0.5 ± 0.6*	−18 ± 2	+17 ± 2	90 ± 26	0.27 ± 0.07
(5)	137 − 24	+1.9 ± 0.3	−19 ± 2	+20 ± 2	96 ± 29	0.37 ± 0.09*
	24 − 24	+1.2 ± 0.4*	−19 ± 2	+21 ± 2	98 ± 29	0.38 ± 0.10*

Table 5. Effect of submucosal bumetanide on cellular electrical properties in stimulated monolayers[a]

Condition	ψ_t (mV)	ψ_a (mV)	R_t (Ω cm^2)	f_R
Baseline	+1.2	−25	244	0.30
	±1.0	± 2	±74	±0.03
Bumetanide	+0.9	−30	250	0.34*
(10^{-4} M)	±1.0	± 4	±75	±0.03

[a] Monolayers were stimulated with isoproterenol (5×10^{-6} M) added to the submucosal bathing solution. Values represent results from five monolayers; baseline and experimental values were taken during a single cell impalement in each monolayer. *$P < 0.05$ compared to baseline.

Table 6. Effect of submucosal ouabain on cellular electrical properties in stimulated monolayers[a]

Condition	ψ_t (mV)	ψ_a (mV)	R_t (Ω cm^2)	f_R
Baseline	+3.5	−32	269	0.32
	+0.4	± 2	±93	±0.07
Ouabain	+1.7*	−26*	290	0.33
(10^{-4} M)	±0.4	± 2	±117	±0.07

[a] Monolayers were stimulated with isoproterenol (5×10^{-6} M) added to the submucosal bathing solution. Values represent results from seven monolayers; baseline and experimental values were measured during a single cell impalement in each monolayer. Experimental values were taken 8 to 20 min after addition of ouabain (i.e. for as long as the impalement was stable). ψ_t continued to fall to zero after the end of the impalement. *$P <$ 0.05.

alterations of R_t or f_R, they suggest a minimal cellular or paracellular Na conductance. After adding isoproterenol, we observed similar changes in ψ_t during the ion substitutions. However, we also found that a decrease in submucosal [Na] increased f_R. The best explanation for the increase in f_R is a decrease in basolateral Cl entry into the cell, with a subsequent increase in the resistance of the Cl-permeable apical cell membrane. However, the lack of a statistically significant hyperpolarization of ψ_a does weigh somewhat against this possibility.

Effect of Transport Inhibitors

To further understand the mechanism of ion transport we examined the effect of a few of the agents known to inhibit Cl secretion in the intact tracheal epithelium. Loop diuretics, such as fuorsemide and bumetanide inhibit the electrically neutral Cl entry step at the basolateral membrane (Welsh, 1983*c*; Widdicombe et al., 1983). Table 5 shows that submucosal bumetanide (10^{-4} M) tended to hyperpolarize ψ_a ($P < 0.1$) and increase f_R, changes similar to those previously observed with furosemide (Welsh, 1983*c*) and bumetanide (*unpublished observation*) in intact tracheal epithelium. The most likely explanation for the increase in f_R is the same as that given above for the effect of a decrease in submucosal [Na]: a decrease in Cl entry with a subsequent decrease in intracellular [Cl] would tend to increase R_a and thus f_R.

Table 6 shows that addition of ouabain (10^{-4} M) to the submucosal solution depolarized both the transepithelial and apical membrane voltage without altering R_t or f_R. These changes are the same as those observed in the intact epithelium (Welsh, 1983*b*) and are consistent with inhibition of the basolateral Na pump.

The basolateral membrane K permeability in tracheal epithelium is blocked by submucosal Ba (Welsh, 1983*a*; Smith & Frizzell, 1984), leading to a depolarization of the cell and a decreased rate of Cl secretion. Table 7 shows a similar response in the cultured monolayers: Ba depolarized both ψ_t and ψ_a, suggesting a decreased rate of secretion. Although Ba increased R_t and decreased f_R in the intact epithelium, these values were not significantly

changed in the monolayers. The failure to observe an increase in R_t in the cultured monolayers may be due to a lower ratio of paracellular-to-cellular conductance than in the intact epithelium. The lack of a significant fall in f_R may result from the fact that f_R is already quite low under baseline conditions, thereby minimizing the effect of an increase in basolateral membrane resistance.

Discussion

The results of this study show that primary cultures of canine tracheal epithelium maintain many of the morphologic characteristics and ion transport functions of the intact epithelium. I will consider the similarities and differences between the intact epithelium and the cultured monolayers.

The most important finding is that the monolayers maintain the Cl secretory capacity of the intact epithelium and that the cellular mechanism of Cl secretion is the same. This conclusion is based on the following evidence. 1) Isoproterenol increased ψ_t and decreased R_t, a pattern consistent with the induction of electrogenic transepithelial Cl secretion. 2) Isoproterenol, depolarized ψ_a and decreased apical membrane resistance, changes that suggest an increase in apical Cl permeability similar to that seen in the intact epithelium. 3) Ion substitution studies indicate that secretagogue increases the Cl conductance of the apical membrane and that the basolateral membrane is K conductive under both secreting and nonsecreting conditions. 4) Agents that inhibit specific membrane transport processes in the intact epithelium have similar effects in the cultured cell monolayers: a) submucosal bumetanide has an effect consistent with inhibition of the electrically neutral basolateral Cl entry step; b) submucosal ouabain depolarizes ψ_t and ψ_a without altering R_t or f_R, changes suggesting an inhibition of the basolateral Na pump as is observed in the intact trachea; and c) submucosal Ba depolarizes the cell, a response expected if it interferes with the basolateral K conductance as has been previously observed. Although there are quantitative differences between the response of the cultured monolayers and the intact epithelium to ion substitutions and transport inhibitors, they are, for the most part, qualitatively similar suggesting shared mechanisms of transport. 5) Stimulation of secretion appears to increase both apical and basolateral conductance, as it does in the intact epithelium. These findings all support a generally accepted model of epithelial Cl secretion that applies to trachea as well as to a diverse group of other secretory epithelia (Frizzell et al., 1979; Welsh et al., 1982).

Table 7. Effect of submucosal barium on cellular electrical properties in stimulated monolayers[a]

Condition	ψ_t (mV)	ψ_a (mV)	R_t (Ω cm^2)	f_R
Baseline	+2.0	−28	174	0.34
	±1.3	± 3	±76	±0.05
Ba	+0.2*	−21*	162	0.32
(2×10^{-3} M)	±0.6	± 4	±74	±0.05

[a] Monolayers were stimulated with isoproterenol (5×10^{-6} M) added to the submucosal bathing solution. Values represent results from four monolayers; baseline and experimental values were measured during a single cell impalement in each monolayer. *$P < 0.05$.

The results also indicate that the cultured monolayers retain the capacity for Na absorption, as indicated by the response to amiloride and the formation of domes when the cells were grown on impermeable supports. Although the rate of Na absorption in intact epithelium is usually substantially less than the rate of Cl secretion, in the monolayers the rate of amiloride-sensitive Na absorption was frequently undetectable.

The morphology of the cultured cells shared many features of the intact epithelium. Most prominent were the presence of tight junctions and the differentiation of the two-cell membranes, the apical membrane showed microvilli and the basolateral membrane contained a moderately interdigitated lateral intercellular space. In addition, the appearance of the endoplasmic reticulum and other cytoplasmic organelles was similar to that observed in intact ciliated and basal epithelial cells. The cell population was also much more homogeneous in the cultured cells: I did not observe the goblet cells that are seen in the surface epithelium of intact tissue.

There were two main differences between the monolayers and intact epithelium. First, the cells in the intact epithelium are columnar, while in the monolayers they are flatter and cuboidal. This likely results from the interaction of the cells with the substrate. In other systems, changing the substrate to a floating collagen gel (Emerman & Pitelka, 1977) allows the cells to assume a shape more characteristic of that found in intact epithelium. Second, in monolayers the values of ψ_t and I_{sc} (calculated) were much lower than in the intact epithelium. This difference may relate to differences in morphology: when cells assume a flatter shape, the cell density per cm^2 will be much lower and thus the net rate of transport may be lower. This speculation assumes that intact epithelial cells and cultured cells have a similar density of ion transport proteins (channels,

carriers, and pumps) per cell. The validity of this assumption is unknown. Moreover, it is not known whether the electrical resistance of a unit length of tight junction is the same in intact and cultured epithelia.

One minor difference between the cultured cells and the intact epithelium is that we did not see changes in ψ_t or R_t following addition of indomethacin. Indomethacin minimizes the rate of Cl secretion in the intact epithelium by decreasing the rate of endogenous prostaglandin production and intracellular cAMP levels (Al-Bazzaz et al., 1981; Smith et al., 1982). Although the cultured monolayer may have a different rate of prostaglandin production, it is possible that we missed an effect of indomethacin because the baseline levels (and changes) in ψ_t and R_t are small in the monolayers, and the time course of the response to indomethacin is slow (20 to 60 min) in the intact epithelium.

There have been several previous reports demonstrating that culture of epithelial cells on permeable supports allows them to function as transporting epithelia. However, most studies have focused on Na-absorbing epithelia (for example *see:* Handler et al., 1980; Valentich, 1981; Sakhrani & Fine, 1983). There have only been a few studies in Cl-secreting epithelia. In a previous report, Coleman et al. (1984) reported that cultured tracheal epithelium grown on permeable supports developed a transepithelial resistance and responded to a variety of secretagogues with an increase in short-circuit current. In addition, the current was inhibited by submucosal ouabain and bumetanide, suggesting that the current resulted from Cl secretion. In the present studies we used methods almost identical to those of Coleman et al. (1984); however, our values of ψ_t and R_t are somewhat lower than those they report for monolayers obtained from two animals. Although the reason for this difference is unknown, it might have resulted from a greater contribution of edge-damage in our study (as we used a smaller chamber), differences in fluid flow across the monolayer (we used an impalement chamber while they used Ussing chambers), or differences in the bathing solutions (we used HCO_3-Ringer's while they used the culture media). Dharmsathaphorn et al. (1984) have also reported that a colonic tumor cell line maintains the capacity for Cl secretion in culture. Finally, in the Madin Darby Canine Kidney cell line (MDCK), Brown and Simmons (1981) have demonstrated that epinephrine stimulates Cl secretion.

The advantage of this study, in comparison to previous studies of cultured cells, is that we have been able to apply intracellular microelectrode techniques to the study of ion transport. We have found, with a variety of experimental maneuvers, that the two individual cell membranes contain the same ion transport processes as the intact epithelium and that intact and cultured epithelia share a common cellular mechanism of Cl secretion. Thus, we can conclude that even though the overall rate of transepithelial transport is lower in monolayers of cultured cells, each cell contains the same ion transport "machinery" as cells in the intact epithelium.

In summary, these results indicate that primary cultures of canine tracheal epithelium may prove valuable in further investigations of the mechanism and regulation of Cl secretion.

The author especially wishes to thank Drs. Jonathan H. Widdicombe and Diana L. Coleman for their help and discussions when the cell culture was begun. The help of Rick Roberts in preparing the specimens for freeze-fracture electron microscopy and the technical assistance of Timothy R. Ruppert and Phil Karp are greatly appreciated. The secretarial assistance of Mary Uhl, Deanna Ollendick, and Ann Ziskovsky is also appreciated.

This work was supported by research grants from the National Heart Lung and Blood Institute (HL-29851) and the Cystic Fibrosis Foundation. Dr. Welsh is an Established Investigator of the American Heart Association.

References

Al-Bazzaz, F., Yadava, V.P., Westenfelder, C. 1981. Modification of Na and Cl transport in canine tracheal mucosa by prostaglandins. *Am. J. Physiol.* **240:**F101–F105

Brown, C.D.A., Simmons, N.L. 1981. Catecholamine-stimulation of Cl^- secretion in MDCK cell epithelium. *Biochim. Biophys. Acta* **649:**427–435

Coleman, D.L., Tuet, I.K., Widdicombe, J.H. 1984. Electrical properties of dog tracheal epithelial cells grown in monolayer culture. *Am. J. Physiol.* **246:**C355–C359

Dharmsathaphorn, K., McRoberts, J.A., Mandel, K.G., Tisdale, L.D., Masui, H. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* **246:**G204–G208

Diamond, J.M. 1982. Transcellular cross-talk between epithelial cell membranes. *Nature (London)* **300:**683–685

Emerman, J.T., Pitelka, D.R. 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In Vitro* **13:**316–328

Frasca, J.M., Auerbach, O., Parks, V.R., Jamieson, J.D. 1968. Electron microscopic observations of the bronchial epithelium of dogs. I. Control dogs. *Exp. Mol. Pathol.* **9:**363–379

Frizzell, R.A., Field, M., Schultz, S.G. 1979. Sodium-coupled chloride transport by epithelial tissues. *Am. J. Physiol.* **236:**F1–F8

Greger, R., Schlatter, E. 1984. Mechanism of NaCl secretion in the rectal gland of spiny dogfish (*Squalus acanthias*). I. Experiments in isolated *in vitro* perfused rectal gland tubules. *Pfluegers Arch.* **402:**63–75

Handler, J.S., Perkins, F.M., Johnson, J.P. 1980. Studies of renal cell function using cell culture techniques. *Am. J. Physiol.* **238:**F1–F9

Marin, M.L., Lane, B.P., Gordon, R.E., Drummon, E. 1979. Ultrastructure of rat tracheal epithelium. *Lung* **156:**223–236

Nagel, W., Reinach, P. 1980. Mechanism of stimulation by epinephrine of active transepithelial Cl transport in isolated frog cornea. *J. Membrane Biol.* **56:**73–79

Rhodin, J.A.G. 1966. Ultrastructure and function of the human tracheal mucosa. *Am. Rev. Respir. Dis.* **93:**1–15

Sakhrani, L.M., Fine, L.G. 1983. Renal tubular cells in culture. *Mineral Electrolyte Metab.* **9:**276–281

Schultz, S.G. 1981. Homocellular regulatory mechanisms in sodium-transporting epithelia: Avoidance of extinction by "flush-through." *Am. J. Physiol.* **241:**F579–F590

Shorofsky, S.R., Field, M., Fozzard, H.A. 1983. Electrophysiology of Cl secretion in canine trachea. *J. Membrane Biol.* **72:**105–115

Shorofsky, S.R., Field, M., Fozzard, H.A. 1984. Mechanism of Cl secretion in canine trachea: Changes in intracellular chloride activity with secretion. *J. Membrane Biol.* **81:**1–8

Smith, P.L., Frizzell, R.A. 1984. Chloride secretion by canine tracheal epithelium: IV. Basolateral membrane K permeability parallels secretion rate. *J. Membrane Biol.* **77:**187–199

Smith, P.L., Welsh, M.J., Stoff, J.S., Frizzell, R.A. 1982. Chloride secretion by canine tracheal epithelium: I. Role of intracellular cAMP levels. *J. Membrane Biol.* **70:**217–226

Sugahara, K., Caldwell, J.H., Mason, R.J. 1984. Electrical currents flow out of domes formed by cultured epithelial cells. *J. Cell Biol.* **99:**1541–1546

Tanner, C., Frambach, D.A., Misfeldt, D.S. 1983. Transepithelial transport in cell culture. *Biophys. J.* **43:**183–190

Valentich, J.D. 1981. Morphological similarities between the dog kidney cell line MDCK and the mammalian cortical collecting tubule. *In:* Hormonal Regulation of Epithelial Transport of Ions and Water. W.N. Scott and D.B.P. Goodman, editors. Vol. 372, pp. 384–404. *Ann. N.Y. Acad. Sci.*, New York

Welsh, M.J. 1983*a*. Barium inhibition of basolateral membrane potassium conductance in tracheal epithelium. *Am. J. Physiol.* **244:**F639–F645

Welsh, M.J. 1983*b*. Evidence for a basolateral membrane potassium conductance in canine tracheal epithelium. *Am. J. Physiol.* **244:**C377–C384

Welsh, M.J. 1983*c*. Inhibition of chloride secretion by furosemide in canine tracheal epithelium. *J. Membrane Biol.* **71:**219–226

Welsh, M.J. 1983*d*. Intracellular chloride activities in canine tracheal epithelium: Direct evidence for sodium-coupled intracellular chloride accumulation in a chloride-secreting epithelium. *J. Clin. Invest.* **71:**1392–1401

Welsh, M.J., Smith, P.L., Frizzell, R.A. 1982. Chloride secretion by canine tracheal epithelium: II. The cellular electrical potential profile. *J. Membrane Biol.* **70:**227–238

Welsh, M.J., Smith, P.L., Frizzell, R.A. 1983. Chloride secretion by canine tracheal epithelium: III. Membrane resistances and electromotive forces. *J. Membrane Biol.* **71:**209–218

Widdicombe, J.H., Nathanson, I.T., Highland, E. 1983. Effects of "loop" diuretics on ion transport by dog tracheal epithelium. *Am. J. Physiol.* **245:**C388–C396

Yoneda, K. 1976. Mucous blanket of rat bronchus. *Am. Rev. Respir. Dis.* **114:**837–842

Received 4 March 1985; revised 5 August 1985