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Intracellular chloride activities in canine tracheal epithelium. Direct evidence for sodium-coupled intracellular chloride accumulation in a chloride-secreting epithelium.

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Research Article

Canine tracheal epithelium secretes Cl via an electrogenic transport process that appears to apply to a wide variety of secretory epithelia. To examine the mechanisms involved, intracellular chloride activity, acCl, was measured with Cl-selective intracellular microelectrodes. The results indicate that when the rate of secretion was minimal acCl was 37 mM; with stimulation of secretion the intracellular voltage depolarized, but acCl was not significantly altered, at 39 mM. These findings indicate that: (a) Cl is accumulated across the basolateral membrane under nonsecreting and secreting conditions at an activity 3.8 and 2.4 times, respectively, that predicted for an equilibrium distribution; (b) Cl exit across the apical membrane may be passive with an electrochemical driving force of 22 mV; and (c) stimulation of secretion enhanced the rate of Cl entry across the basolateral membrane, since Cl transport increased without a change in acCl. In the absence of Na in the extracellular fluid, acCl approached the value expected for an equilibrium distribution. This finding suggests that "uphill" entry of Cl into the cell against its electrochemical gradient is dependent upon, and energized by, the entry of Na down its gradient. Submucosal bumetanide, a loop diuretic, also decreased the rate of Cl secretion and decreased acCl, indicating an inhibition of Cl entry. These findings indicate that Cl entry into the cell is directed against […]

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Intracellular Chloride Activities in Canine Tracheal Epithelium

DIRECT EVIDENCE FOR SODIUM-COUPLED INTRACELLULAR CHLORIDE ACCUMULATION IN A CHLORIDE-SECRETING EPITHELIUM

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ABSTRACT Canine tracheal epithelium secretes Cl via an electrogenic transport process that appears to apply to a wide variety of secretory epithelia. To examine the mechanisms involved, intracellular chloride activity, a_c^{Cl}, was measured with Cl-selective intracellular microelectrodes. The results indicate that when the rate of secretion was minimal a_c^{Cl} was 37 mM; with stimulation of secretion the intracellular voltage depolarized, but a_c^{Cl} was not significantly altered, at 39 mM. These findings indicate that: (*a*) Cl is accumulated across the basolateral membrane under nonsecreting and secreting conditions at an activity 3.8 and 2.4 times, respectively, that predicted for an equilibrium distribution; (*b*) Cl exit across the apical membrane may be passive with an electrochemical driving force of 22 mV; and (*c*) stimulation of secretion enhanced the rate of Cl entry across the basolateral membrane, since Cl transport increased without a change in a_c^{Cl}.

In the absence of Na in the extracellular fluid, a_c^{Cl} approached the value expected for an equilibrium distribution. This finding suggests that "uphill" entry of Cl into the cell against its electrochemical gradient is dependent upon, and energized by, the entry of Na down its gradient. Submucosal bumetanide, a loop diuretic, also decreased the rate of Cl secretion and decreased a_c^{Cl}, indicating an inhibition of Cl entry. These findings indicate that Cl entry into the cell is directed against its electrochemical gradient and is mediated by a Na-coupled, bumetanide-inhibitable, transport process at the basolateral membrane and that Cl may exit passively down a favorable electrochemical gradient across the apical membrane.

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INTRODUCTION

Ion transport by the airway epithelium is an important process controlling the quantity and composition of the respiratory tract fluid and, thus, in effecting normal pulmonary mucociliary clearance. In vitro canine tracheal epithelium actively secretes Cl and absorbs Na (1) via electrogenic transport processes that result in a transepithelial electrical potential difference (ψ_t) of 20–40 mV with the submucosal solution electrically positive with respect to the mucosal solution. The rate of Cl secretion is controlled by a variety of agents that mediate intracellular levels of cAMP and calcium (2).

Transepithelial Cl secretion is inhibited by removal of Na from the submucosal bathing solution (3, 4) and by inhibition of the basolateral membrane Na-K-ATPase by submucosal ouabain or removal of K from the submucosal bathing media (3–6). These findings suggested that Cl enters the cell across the basolateral membrane via a NaCl cotransport process and is recycled back across the basolateral membrane by the Na pump. Such a process would explain both the dependence of Cl secretion on submucosal Na and the inhibition of Cl secretion by ouabain and K-free media.

The recent application of intracellular microelectrode techniques to the problems of Cl secretion in tracheal epithelium has provided further support for this proposed mechanism of Cl secretion. Cl entry across the basolateral membrane appears to be electrically neutral, as indicated by (*a*) the failure of removal of Cl from the submucosal solution to alter the electrical potential difference across the basolateral membrane (ψ_b) or the relative resistance of the basolateral membrane (7); and (*b*) submucosal furosemide (10^{-3} M), a loop diuretic, inhibited Cl secretion with-

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out altering the basolateral membrane resistance or electromotive force, indicating an inhibition of neutral Cl entry (8). An equivalent electrical circuit analysis of the mechanism of Cl secretion also suggested that Cl is accumulated intracellularly (9) and that submucosal furosemide decreases the intracellular Cl concentration (8). These observations, taken together, are consistent with a basolateral membrane NaCl cotransport process.

The purpose of this study was to measure the thermodynamic activity of intracellular Cl, a_c^{Cl}, in canine tracheal epithelium using intracellular ion-selective microelectrodes to directly establish the mechanism of Cl secretion and the validity of the hypothesis given above. The specific aims of this study were to: (*a*) determine the effect of the rate of Cl secretion on a_c^{Cl}; (*b*) to evaluate the role of the Na gradient in intracellular Cl accumulation and transepithelial transport; and (*c*) to determine the effect of loop diuretics on Cl entry.

GLOSSARY

ψ_t, ψ_a, ψ_b	electrical potential difference across the epithelium, the apical membrane, and the basolateral membrane, respectively, in millivolts.
ψ^{Cl}	electrical potential difference across the apical membrane measured by the Cl-selective microelectrode, in millivolts.
I_{sc}	short circuit current, i.e., the current required to clamp ψ_t to zero, in $\mu A \cdot cm^{-2}$.
R_t	transepithelial resistance, in $\Omega \cdot cm^2$.
a_c^{Cl}, a_m^{Cl}, a_s^{Cl}	Cl activity in the cell interior, the mucosal solution, and submucosal solution, respectively, in millimolars.
$\Delta\tilde{\mu}_a^{Cl}$, $\Delta\tilde{\mu}_b^{Cl}$	electrochemical driving force for Cl at the apical and basolateral cell membranes, respectively, in millivolts.
$\Delta\tilde{\mu}_b^{Na}$	electrochemical driving force for Na at the basolateral membrane, in millivolts.
f_R	fractional resistance of the apical cell membrane.

METHODS

Tissues and solutions. Tracheal epithelium was prepared as previously described (10). Mongrel dogs (25–40 kg) of either sex were anesthetized with pentobarbital (25 mg/kg i.v.) and the trachea was removed. The muscular layer was removed from the posterior membranous portion of the trachea and the epithelium was mounted, mucosal surface up, as a flat sheet between two halves of a lucite micropuncture chamber (0.125 cm^2 aperture) (7). Both the mucosal and submucosal surfaces of the epithelium were continuously perfused by gravity flow from reservoirs above the chamber at a rate of 2 $ml \cdot min^{-1}$. The perfusion reservoir was water-jacketed so that the solution bathing the tissue was maintained at 37°C.

The transepithelial electrical potential difference across the epithelium (ψ_t) was measured by an automatic voltage-current clamp (University of Iowa, Department of Bioengineering) via two calomel half-cells connected to the outflow tract of the chamber with saturated KCl bridges. The mucosal solution was used as reference for all electrical measurements. The voltage clamp was also used to pass sufficient current across the epithelium (via Ag-AgCl rings implanted in the chamber) to clamp ψ_t to zero, i.e., the short circuit current (I_{sc}) condition. Transepithelial electrical resistance (R_t) was calculated from the change in current required to intermittently clamp ψ_t to ±10–15 mV (pulse duration 1–2 s; interval 10 s). ψ_t and I_{sc} were recorded on a strip chart recorder (Gould Inc., Cleveland, OH).

The bathing solution contained (in millimolars): 118.9 NaCl, 20.4 $NaHCO_3$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose. The solution was bubbled with 95% O_2 and 5% CO_2 (pH 7.4 at 37°C). For Cl-free solutions, Na gluconate was substituted for NaCl, $CaSO_4$ for $CaCl_2$, and $MgSO_4$ for $MgCl_2$. For Na-free solutions, choline was substituted for Na. Indomethacin (10^{-6} M) (Sigma Chemical Co., St. Louis, MO) was added to the mucosal bathing solution of some tissues to minimize the basal rate of Cl secretion and thus to maximize the change observed during the subsequent stimulation of Cl secretion. Mucosal addition of indomethacin decreases the endogenous rate of prostaglandin production, decreases intracellular cAMP, and thus decreases the rate of Cl secretion without interfering with the subsequent response to secretagogues (2, 11). Epinephrine (10^{-6} M, submucosal solution) (Elkins-Sinn, Inc., Cherry Hill, NJ) was used to stimulate Cl secretion (7). Bumetanide (10^{-4} M) (a loop diuretic structurally and functionally similar to furosemide, 12) was added to the submucosal bathing solution to inhibit Cl secretion.

Intracellular microelectrodes. Conventional, potential-measuring microelectrodes and chloride-selective microelectrodes were prepared from 1.2 mm OD borosilicate, fiber-filled glass capillaries (W. P. Instruments, New Haven, CT). The glass was cleaned by a 4-h immersion in concentrated nitric acid, rinsed overnight in distilled water, and then dried at 70°C. The electrodes were pulled on a horizontal micropipette puller (either a model P-77, Sutter Instrument Co., San Francisco, CA; this puller was used for the majority of the study; or an M1 Industrial Science Associates, Inc., Ridgewood, NY).

The conventional electrodes were then backfilled with 0.5 M KCl and had a tip resistance of 85–130 MΩ when the tip was immersed in 0.5 M KCl (7, 13). The tips of chloride-selective microelectrodes were submerged in a filtered solution of 1% Glasclad (Petrarch Systems Inc., Levittown, PA) in 1-chloronaphthelene and a vacuum was applied to the open end for 50–60 min. The electrodes were then baked at 100°C for 1 h. The tip of the electrode was then immersed in liquid Cl exchanger (477913, Corning Medical, Medfield, MA) (14) and suction applied for another 50–60 min. Following this the shaft and body of the electrode were backfilled with 0.5 M KCl and the tip was immersed in 0.5 M KCl for at least 1 h. Electrodes were used within 20 h of fabrication. Cl-selective microelectrodes had a resistance of $8.6 \pm 1.7 \times 10^{10}\Omega$ (n = 24 microelectrodes).

Calibration of Cl-selective electrodes. Cl-selective electrodes were calibrated before (and usually after) each experiment using published techniques and formulae (15). The calibration solutions included 25-, 50-, and 150-mM solutions of KCl and the normal Ringer's solution. Ion activities were calculated from published activity coefficients (16). The electrical circuit used for calibrating the electrodes was identical to that used during cellular impalements. The mean slope of the line relating the electrical potential measured by the chloride electrode and the natural logarithm of the Cl activity in the calibrating solution was a straight line with a slope (S) of −23.9±0.3 mV at the ambient room temperature. The measured slope compares with an ideal slope (RT/F) of −25.6±0.03 mV where R, T, and F have their usual meanings. The electrodes were calibrated at room temperature

and used at 37°C. Comparison of S measured at room temperature and at 37°C in three electrodes indicated that the ratio of the measured S to the predicted ideal S was not significantly different at the two temperatures. Therefore, the slope used in calculation of the intracellular Cl activity was taken as the slope measured at room temperature multiplied by the ratio of the predicted ideal slope at 37°C to the predicted ideal slope at the ambient room temperature. This resulted in an increase in the value of S of 4%.

The electrical potential measured by the chloride electrode may be influenced by the presence of intracellular anions other than Cl. In this regard, HCO_3 is the only anion likely to have a high enough intracellular activity that it would interfere substantially with the measurement of a_c^{Cl}. Calibration of chloride electrodes in $NaHCO_3$ solutions revealed a slope of -11.1 ± 0.6 mV compared with a predicted ideal slope of -25.7 mV. Thus, the selectivity coefficient for Cl vs. HCO_3 varied with the ionic strength of the calibrating solutions. At equal Cl and HCO_3 activities of 93 mM (the a^{Cl} of the Ringer's solution), $k_{Cl,HCO_3} = 0.077$ corresponding to a Cl:HCO_3 selectivity of 13.9:1. At Cl and HCO_3 activities of 30 mM, $k_{Cl,HCO_3} = 0.145$ with a Cl:HCO_3 selectivity of 7.4:1. Since the intracellular HCO_3 activity is not likely to exceed that in the extracellular medium it can be shown that the effect of HCO_3 on the chloride electrodes is unlikely to alter the estimate of a_c^{Cl} by >1–2 mM. Finally, the Cl activity measured in the Ringer's solution was not significantly different from the value of 93 mM calculated for a NaCl solution with the ionic strength of the normal Ringer's solution.

Intracellular recordings. The microelectrode tips were advanced into the epithelium with a hydraulic microdrive (model 8, Narishige, Japan). The electrical potential difference across the apical membrane (ψ_a), with reference to the mucosal solution, was measured with a high input impedance ($10^{15}\Omega$) electrometer (model FD223, W. P. Instruments Inc.) and recorded on a strip chart recorder (Gould, Inc.). The criteria for a successful impalement were the same as those previously used (7): (*a*) an abrupt negative deflection; (*b*) a stable plateau value whose magnitude was at least as large as the initial deflection; (*c*) a stable (±2 mV) plateau value of ψ_a for at least 30 s; and (*d*) an abrupt return to the base line (preimpalement) value (±2 mV) upon withdrawal of the electrode. The electrical potential difference across the apical membrane measured with Cl-selective microelectrodes (ψ^{Cl}) was determined using the same impalement criteria and apparatus used for conventional electrodes. Two to seven successful determinations of ψ_a and ψ^{Cl} were made under each experimental condition in each tissue. The mean values of ψ_a and ψ^{Cl} were then used to calculate the intracellular Cl activity a_c^{Cl}, from the relation:

$$\psi^{Cl} = \psi_a + S \ln\left(\frac{a_c^{Cl}}{a_m^{Cl}}\right), \quad (1)$$

where a_m^{Cl} refers to the Cl activity in the mucosal bathing solution. This calculation assumes no interference from other intracellular anions.

Results are presented as means±SEM. Statistical significance was evaluated using a paired t test; a P value < 0.05 was considered statistically significant.

RESULTS

Cl activities in NaCl Ringer's solution. Fig. 1 shows an example of a successful cellular impalement with a Cl-selective microelectrode before and after the addition of epinephrine (10^{-6} M). Submucosal addition of epinephrine produced an increase in the rate of Cl secretion as indicated by the increase in I_{sc}. There was also a decrease in the transepithelial resistance, R_t, as indicated by the increase in the magnitude of the bipolar current pulses required to intermittently clamp ψ_t to ±10 mV. Stimulation of secretion also produced a transient, biphasic response in the value of the electrical potential difference across the apical membrane measured with a chloride electrode, ψ^{Cl}, and the fractional resistance of the apical membrane, f_R:

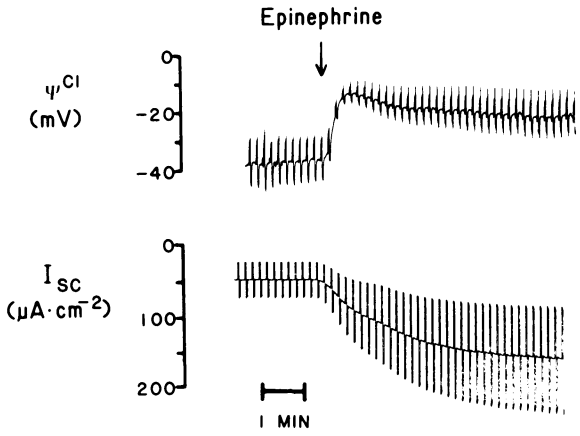

FIGURE 1 Recording of the acute electrical response to stimulation of secretion obtained with a Cl-selective microelectrode. The arrow indicates the onset of the electrical response to addition of epinephrine (10^{-6} M, submucosal solution). The biphasic pattern of the changes in ψ^{Cl} and f_R is similar to that observed with conventional (KCl filled) microelectrodes (7, 9).

$$f_R = \frac{\Delta\psi_a}{\Delta\psi_t} = \frac{R_a}{R_a + R_b}, \quad (2)$$

where $\Delta\psi_a$ and $\Delta\psi_t$ refer to the changes in ψ_a and ψ_t that result from transepithelial current pulses and R_a and R_b are the resistances of the apical and basolateral membranes, respectively. Fig. 1 shows that with addition of secretagogue ψ^{Cl} rapidly depolarized and then partially repolarized over the course of 2–3 min. A biphasic response in f_R is also apparent from the initial decrease in the magnitude of the voltage deflection across the apical membrane produced by the current pulses followed by a secondary increase in the magnitude of the voltage deflections. This biphasic pattern of f_R and the electrical potential difference across the apical membrane is a characteristic response to stimulation of secretion in canine tracheal epithelium (7, 9). The biphasic behavior of f_R and intracellular voltage is produced by an initial increase in the Cl permeability of the apical cell membrane (7, 9) followed by a secondary increase in the K permeability of the basolateral membrane (9, 17). The observation that the biphasic pattern observed with Cl-selective electrodes is the same as that previously observed with

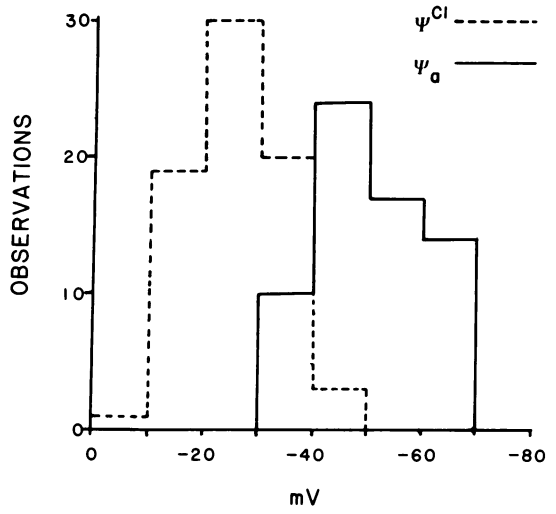

FIGURE 2 Histogram of the value of membrane potential obtained with conventional (ψ_a, solid line) and Cl-selective microelectrodes (ψ^{Cl}, dashed line). Values were obtained in tissues bathed in the regular NaCl Ringer's in which Cl secretion was stimulated with epinephrine (10^{-6} M, submucosal solution).

conventional electrodes indicates that: (*a*) impalements with conventional and Cl-selective electrodes are comparable in quality; and (*b*) suggests the absence of large changes in intracellular Cl activity. For the studies reported below, transepithelial and intracellular measurements were obtained only during the steady state, before or after an experimental intervention.

Fig. 2 is a histogram of the values of ψ_a and ψ^{Cl} obtained in all tissues bathed with NaCl Ringer's, under secreting conditions (epinephrine, 10^{-6} M, submucosal solution). Both ψ_a and ψ^{Cl} are distributed normally; there is no evidence for more than one population of cells.

The data obtained in seven tissues under both nonsecreting (indomethacin, 10^{-6} M, mucosal solution) and secreting conditions (epinephrine, 10^{-6} M, submucosal solution) is shown in Table I.[1] When the rate of Cl secretion was minimal, a_c^{Cl} was 37 mM, compared to the value of 10 mM expected for an equilibrium distribution of Cl (a_{eq}^{Cl}), based on the value of ψ_a. Thus, Cl is accumulated in the cell at an activity 3.8 times greater than predicted for electrochemical equilibrium. After addition of epinephrine, I_{sc} increased, R_t decreased, and ψ_a depolarized reflecting an increase in the rate of electrogenic Cl secretion. Despite these changes, a_c^{Cl} was unaltered. The electrochemical driving force ($\Delta\tilde{\mu}_a^{Cl}$) for Cl movement across the apical membrane (in millivolts) was calculated as:

$$\Delta\tilde{\mu}_a^{Cl} = \psi_a + \frac{RT}{zF}\ln\left(\frac{a_c^{Cl}}{a_m^{Cl}}\right). \quad (3)$$

The value of $\Delta\tilde{\mu}_a^{Cl}$ is exactly equal and opposite to the electrochemical driving force for Cl movement across the basolateral membrane ($\Delta\tilde{\mu}_b^{Cl}$) under short circuit conditions.[2] $\Delta\tilde{\mu}_a^{Cl}$ decreased significantly from -35 ± 3 mV under nonsecreting conditions to -22 ± 2 mV during stimulation of secretion with epinephrine ($P < 0.001$). This decrease in driving force is entirely accounted for by the depolarization of ψ_a and ψ_b.

The fractional resistance of the apical membrane, f_R (Eq. 2), decreased with stimulation of secretion from 0.86 ± 0.02 to 0.68 ± 0.03 ($P < 0.001$) as previously described, reflecting, in part, a decrease in the resistance of the apical membrane (7, 9). Comparison of the value of f_R obtained with conventional microelectrodes with f_R obtained with Cl-selective microelectrodes for the seven tissues shown in Table I under both nonsecreting and secreting conditions is shown in Fig. 3. There is no systematic difference between the two values, suggesting that the quality of impalements obtained with chloride electrodes is the same as that obtained with conventional electrodes.

Cellular impalements in Cl-free Ringer's solution. Four tissues were bathed in Cl-free, gluconate Ringer's for 1.5–2 h and then cellular impalements were made with both conventional and chloride electrodes. In two of the tissues, impalements were repeated after a return to the normal, Cl containing Ringer's. As shown in Table II, there was a tendency for ψ^{Cl} to exceed ψ_a although the mean difference was not statistically significant. If the cells were completely free of Cl and all interfering anions were at equilibrium across the apical membrane, ψ^{Cl} would be expected to equal ψ_a. The tendency for ψ^{Cl} to exceed ψ_a suggests either the presence of intracellular interfering anions or that there was residual intracellular Cl.[3] Although it was

[1] Table I provides not only the mean values for the group of tissues during the two experimental conditions, but also the mean values obtained in individual tissues. The data is presented in detail to illustrate the consistency of measurements made within individual tissues and the variations observed among different tissues.

[2] Under short circuit conditions, ψ_a is equal and opposite to the electrical potential difference across the basolateral membrane (ψ_b), so that the sum of the two electrical potential differences, arranged in series, yields a ψ_t of zero. Thus $\Delta\tilde{\mu}_a^{Cl}$ is equal and opposite to $\Delta\tilde{\mu}_b^{Cl}$.

[3] The Cl concentrations of the tissue used for cellular impalements could not be accurately determined at low concentrations because of the small size of the tissue. However, the Cl concentration of larger pieces of epithelia (including the submucosal connective tissue) mounted in "Ussing" chambers (1.5 cm^2) and washed with Cl-free Ringer's five times over the course of 1.5 h was not significantly different from zero. Thus, the Cl concentration of the epithelium in the micropuncture chamber, which is continually perfused with fresh Ringer's solution, is probably also near zero.

TABLE I
Intracellular Chloride Activities in the Presence and Absence of Secretion

	I_{sc}	R_t	ψ_a	ψ^{Cl}	a_c^{Cl}	a_{eq}^{Cl}	$\frac{a_c^{Cl}}{a_{eq}^{Cl}}$	$\Delta\bar{\mu}_a^{Cl}$
	$\mu A \cdot cm^{-2}$	$\Omega \cdot cm^2$	*mV*	*mV*	*mM*	*mM*		*mV*
Nonsecreting tissue								
1	14±1	561±6	−59±1 (4)	−34±1 (3)	33	10	3.2	−31
2	44±3	321±16	−64±4 (3)	−44 (1)	38	8	4.5	−40
3	45±1	675±21	−54±1 (3)	−23±6 (6)	29	13	2.3	−22
4	9±1	593±7	−54±1 (3)	−35±1 (4)	44	12	3.6	−34
5	34±1	360±7	−60±1 (4)	−40±1 (4)	41	10	4.1	−38
6	10±1	832±38	−62±1 (3)	−45±3 (5)	48	9	5.3	−45
7	57±1	746±13	−66±1 (4)	−37±6 (3)	30	8	3.8	−36
Mean	30±7	584±71	−60±2	−37±3	37±3	10±1	3.8±0.4	−35±3
Secreting tissue								
1	98±1	262±6	−51±1 (4)	−26±2 (5)	34	14	2.4	−24
2	86±4	217±3	−50±1 (2)	−32±6 (4)	43	15	2.9	−29
3	163±3	184±3	−42±1 (3)	−17±3 (3)	36	20	1.8	−16
4	82±2	288±5	−46±1 (4)	−20±1 (4)	34	17	2.0	−19
5	120±1	191±1	−34±1 (5)	−20±1 (4)	50	25	2.0	−18
6	111±1	303±4	−49±1 (2)	−30±4 (6)	45	15	3.1	−29
7	146±3	322±7	−49±1 (4)	−27±1 (6)	33	15	2.2	−21
Mean	115±11*	252±21*	−46±2*	−24±2*	39±3	17±2*	2.4±0.2*	−22±2*

Values represent the means obtained in seven epithelia when the rate of Cl secretion was minimal, "Nonsecreting" and during the steady state following stimulation of secretion with epinephrine, (10^{-6} M, submucosal solution), "Secreting." Indomethacin (10^{-6} M, mucosal solution) was present under nonsecreting conditions to minimize the spontaneous rate of Cl secretion and remained in the mucosal solution following the addition of epinephrine. The values in parentheses represent the number of impalements with conventional and chloride-selective electrodes. * $P < 0.005$.

not possible to determine the absolute magnitude of such interference, the main point of these experiments is that $\psi^{Cl} \simeq \psi_a$, indicating the lack of a major confounding variable influencing the results.

Intracellular chloride activities in the absence of Na. Table III shows the data obtained in five tissues that had been perfused with Na-free, choline Ringer's for 1.5–2 h and then during the subsequent perfusion with Na-containing Ringer's. In the absence of Na, a_c^{Cl} approached but did not quite reach, the value predicted for an equilibrium distribution based on the value of ψ_a. This small accumulation of Cl above equilibrium may represent interference from other intracellular anions or the failure to achieve a Na concentration of zero in the tissue. In accord with this suggestion is the observation that the I_{sc} did not reach a value of zero. After the switch to Na-containing Ringer's, I_{sc} increased (reflecting an increase in the rate of Cl secretion), R_t decreased, and a_c^{Cl} increased to a value 2.9 times the value expected for an equilibrium distribution. Thus, the important point of this study is that a_c^{Cl} was much decreased in the absence of Na in the bathing solution.

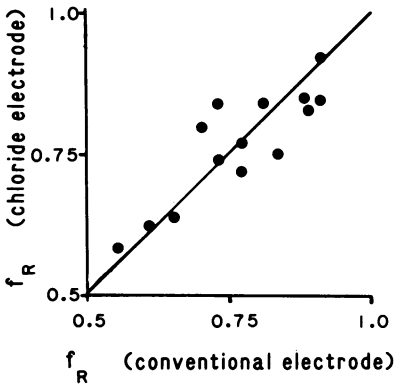

FIGURE 3 Comparison of values of fractional resistance of the apical membrane obtained with conventional and Cl-selective microelectrodes. Values represent the mean values for the tissues shown in Table I under both nonsecreting and secreting conditions. The line shown is the line of identity.

Effect of bumetanide on intracellular chloride activity. To examine the effect of loop diuretics on a_c^{Cl}, I chose to use bumetanide (which is structurally and functionally related to furosemide, 12), rather than furosemide. While both diuretics interact with the chloride exchange resin, thus producing an interfering signal, the interference from bumetanide is less

TABLE II

Electrical Potential Difference Across the Apical Membrane Measured with Conventional (ψ_a) and Cl-selective (ψ^{Cl}) Microelectrodes in the Absence of Extracellular Chloride

Tissue	Gluconate Ringer's		Cl Ringer's		
	ψ_a	ψ^{Cl}	ψ_a	ψ^{Cl}	a_c^{Cl}
	mV				*mM*
8	−54±1 (7)	−62±2 (3)	−50±1 (5)	−30±1 (5)	39
9	−34±3 (6)	−37±1 (3)	—	—	—
10	−65±1 (3)	−64±1 (3)	−52±2 (4)	−16±1 (3)	21
11	−59±3 (4)	−60±9 (2)	—	—	—
Mean	−53±7	−56±6	—	—	—

In two of the tissues the measurements were repeated after returning to the normal Cl-containing Ringer's. Epinephrine (10^{-6} M) was present in the submucosal bathing solution throughout. The values in parentheses indicate the number of impalements.

than that from furosemide.[4] Furthermore, bumetanide is a more potent inhibitor of Cl secretion in canine tracheal epithelium (18), thus allowing the use of 10^{-4} M bumetanide rather than the 10^{-3} M furosemide required to obtain a maximal inhibition of Cl secretion (8, 18). The use of a lower concentration of diuretic will minimize the possibility of interference with the chloride electrode in the determination of a_c^{Cl}.

Table IV shows the effect on a_c^{Cl} of inhibition of Cl secretion with 10^{-4} M bumetanide added to the submucosal bathing solution of three epithelia. Bumetanide decreased the I_{sc} and increased R_t slightly, reflect-

[4] The Cl-selective electrode responded to changes in the concentration of both diuretics. Increasing the furosemide concentration from 10^{-4} M to 10^{-3} M produced a −19±5-mV signal (n = 4), while increasing the bumetanide concentration from 10^{-4} to 10^{-3} M produced a −6±5-mV signal. Since the Cl-selective electrode is less sensitive to bumetanide than furosemide ($P < 0.05$), bumetanide was used for these studies.

TABLE III

Intracellular Chloride Activities in the Absence and Presence of Extracellular Sodium

Tissue	I_{sc}	R_t	ψ_a	ψ^{Cl}	a_c^{Cl}	a_{eq}^{Cl}	$\frac{a_c^{Cl}}{a_{eq}^{Cl}}$	$\Delta\tilde{\mu}_a^{Cl}$
	$\mu A \cdot cm^{-2}$	$\Omega \cdot cm^2$	*mV*	*mV*	*mM*	*mM*		*mV*
Na-free Ringer's								
12	12±1	333±6	−42±1 (4)	−6±1 (2)	18	19	0.9	+2
13	62±4	217±3	−51±1 (5)	−10±1 (6)	18	14	1.3	−7
14	21±1	268±11	−53±1 (6)	−9±1 (6)	19	13	1.4	−10
15	30±1	341±6	−62±1 (4)	−3±1 (4)	9	9	1.0	+1
16	35±2	450±10	−47±1 (3)	−9±2 (5)	21	16	1.3	−6
Mean	32±19	322±39	−51±3	−8±1	17±2	14±2	1.2±0.1	−4±2
Na Ringer's								
12	114±2	240±3	−35±1 (3)	−13±2 (4)	35	25	1.4	−9
13	258±4	181±1	−61±1 (4)	−40±1 (5)	40	9	4.3	−39
14	45±1	306±4	−61±1 (4)	−33±1 (4)	34	10	3.5	−33
15	82±2	307±6	−66±1 (4)	−36±1 (3)	27	8	3.5	−34
16	134±5	318±2	−40±1 (3)	−16±2 (4)	35	21	1.7	−14
Mean	127±36*	270±26*	−53±6	−28±6*	34±2*	14±4	2.9±0.6*	−26±6*

Measurements were obtained after the five epithelia were bathed for 2 h in Na-free, choline Ringer's, and again, during the steady state after epithelia were bathed in the normal Na-containing Ringer's. Epinephrine (10^{-6} M) was present in the submucosal bathing solution during both experimental conditions. The value in parenthesis represents the number of impalements with conventional and chloride-selective electrodes. * $P < 0.05$.

TABLE IV
Effect of Bumetanide on Intracellular Chloride Activities

Tissue	I_{sc}	R_t	ψ_s	ψ^{Cl}	a_c^{Cl}	a_{eq}^{Cl}	$\frac{a_c^{Cl}}{a_{eq}^{Cl}}$	$\Delta\tilde{\mu}_a^{Cl}$
	$\mu A \cdot cm^{-2}$	$\Omega \cdot cm^2$	*mV*	*mV*	*mM*	*mM*		*mV*
Control								
17	111±1	194±1	−59±1 (3)	−28±3 (3)	23	10	2.3	−22
18	120±3	154±3	−62±2 (4)	−28±3 (5)	23	9	2.5	−24
19	118±2	253±7	−52±4 (3)	−22±4 (2)	32	13	2.4	−24
Mean	116±3	200±29	−58±3	−26±2	26±3	11±1	2.4±0.1	−23±1
Bumetanide								
17	63±2	229±6	−59±3 (3)	−16±3 (4)	15	10	1.4	−9
18	88±3	201±11	−58±1 (5)	−18±2 (3)	17	11	1.6	−13
19	49±4	456±56	−64±2 (3)	−13±1 (2)	16	9	1.8	−16
Mean	67±11*	295±81	−60±2	−16±1*	16±1	10±1	1.6±0.1*	−13±2*

Measurements were made during the steady state before and after the addition of bumetanide (10^{-4} M) to the submucosal bathing solution in three epithelia. Epinephrine (10^{-6} M, submucosal solution) was present during both periods to stimulate Cl secretion. * $P < 0.05$.

ing a decrease in Cl secretion as previously observed (8, 18). The decrease in Cl secretion was accompanied by a decrease in ψ^{Cl}, and a_c^{Cl} decreased in each tissue from a mean value 2.4 times greater than the value predicted for an equilibrium distribution to a value 1.6 times greater. $\Delta\tilde{\mu}_a^{Cl}$ also decreased from −23±1 mV to −13±2 mV ($P < 0.05$) following the addition of bumetanide.

DISCUSSION

Intracellular chloride activity. The results of this study clearly demonstrate that Cl is accumulated in canine tracheal epithelial cells at a thermodynamic activity that is two to four times greater than predicted for a passive distribution. The accumulation of Cl is dependent on Na in the bathing media and is inhibited by the loop diuretic, bumetanide. These results provide, for the first time, direct evidence for the "uphill" transport of Cl into canine tracheal epithelial cells against its electrochemical gradient at the basolateral membrane and suggest that Cl entry is coupled to Na. They also suggest that Cl exit from the cell across the apical membrane may be passive, with Cl moving "downhill," with a favorable electrochemical gradient. These results provide compelling evidence for a model of Cl secretion that was first proposed for the shark rectal gland (19) and appears to apply to a wide variety of Cl secreting epithelia (20), including canine tracheal epithelium (7).

The finding that Cl is accumulated in the cell at a thermodynamic activity of 30–40 mM is in excellent agreement with estimates of intracellular Cl activity obtained from equivalent electrical circuit analysis of Cl secretion by tracheal epithelium (8, 9, 21). In those studies a_c^{Cl} was estimated to be 30, 42, and 35 mM, respectively, based on the calculated electromotive force across the apical membrane under secreting conditions. a_c^{Cl} measured in this study is also in good agreement with a Cl concentration of 50 mM determined in isolated tracheal epithelial cells (22). If the activity coefficient for Cl is the same intracellularly as extracellularly, Cl activity would be expected to equal 38 mM, a value identical to that obtained in this study. These results are also consistent with preliminary reports of a_c^{Cl} in two other Cl secreting epithelia: (*a*) in shark rectal gland (23) a_c^{Cl} was reported to be 57 mM, a value five to seven times greater than the value expected for an equilibrium distribution across the basolateral membrane. However, the complicated anatomy of the organ prevented a comprehensive evaluation of the electrochemical gradients at both cell membranes; and (*b*) in isolated frog corneal epithelium (24) a_c^{Cl} was reported to be 29 mM, three times the value predicted for electrochemical equilibrium.

The advantage of the use of Cl-selective intracellular microelectrodes in the evaluation of the mechanism of Cl secretion is that the thermodynamic activity of intracellular Cl is measured directly. Measurement of ion concentrations may reflect inhomogeneity of intracellular Cl distribution and intracellular binding of Cl and are dependent on estimates of the extracellular space. Secondly, a_c^{Cl} was measured in the cells performing active Cl secretion as indicated by the change in intracellular electrical potential, membrane resistance, and membrane electromotive force that accompany changes in the transport rate of the tissue (7, 9), rather than in nontrans-

porting or subepithelial cells that may be present in isolated cell preparations.

A potential disadvantage of the techniques used in this study is that ψ^{Cl} and ψ_a were measured in different cells with different electrodes. Ideally, double-barrel microelectrodes should be used so that ψ^{Cl} and ψ_a are measured at the same time in the same cell. Double-barrel microelectrodes were not used because the larger tip size may produce substantial impalement damage in small, mammalian cells (tracheal epithelial cells are 3–5 μm in width at the apical surface) and because the problem of electrical coupling between the two barrels is serious in fine tip, high resistance electrodes. However, several considerations suggest that the failure to use double-barrel electrodes is not a problem in this study: (*a*) the histogram of values of ψ_a and ψ^{Cl} (Fig. 2) are normally distributed (see also ref. 7) suggesting a single population of impaled cells; (*b*) the acute, biphasic response of ψ_a and f_R to stimulation of secretion observed with chloride electrodes (Fig. 1) is similar to that observed with conventional electrodes (7, 9); (*c*) the value of f_R obtained with conventional and Cl-selective electrodes was similar (Fig. 3) indicating that the quality of cellular impalement obtained with both electrodes is similar; and (*d*) the data was taken during steady-state transport conditions after an experimental intervention so that electrical potentials and ion activities were at a steady state. Another possible problem in the use of Cl-selective microelectrodes is the potential for interference by intracellular anions other than Cl. Three observations indicate that the measurements of a_c^{Cl} are not substantially influenced by other intracellular anions: (*a*) ψ_a and ψ^{Cl} are nearly identical in Cl-free Ringer's solution (Table II); (*b*) a_c^{Cl} measured in Na-free Ringer's is nearly identical to the value predicted for an equilibrium distribution (Table III); and (*c*) HCO_3, the only other anion likely to be present at an appreciable activity, has less selectivity for the chloride electrode than Cl and probably has a lower intracellular than extracellular activity.

Chloride entry at the basolateral membrane. The present data indicate that Cl is accumulated across the basolateral membrane, against its electrochemical gradient, via a Na-dependent mechanism. Removal of Na from the bathing media decreased a_c^{Cl} to a value consistent with an equilibrium distribution across the two cell membranes. This finding, plus the observation that Cl entry is an electrically neutral process (7, 8), provide compelling evidence that Cl entry is coupled to Na entry and that the energy for "uphill" Cl entry is provided by the entry of Na "down" a favorable electrochemical gradient. The electrochemical potential difference for Na across the basolateral membrane, $\Delta\tilde{\mu}_b^{Na}$, is certain to be substantially greater than the $\Delta\tilde{\mu}_b^{Cl}$ of 22 mV. Widdicombe et al. (22) have estimated

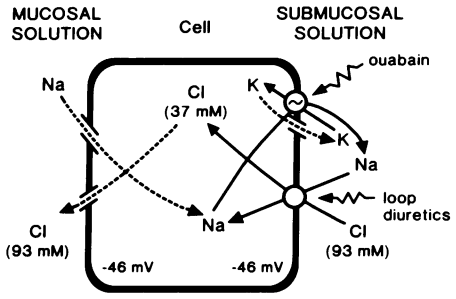

FIGURE 4 A model of chloride secretion by canine tracheal epithelium (see text for details).

an intracellular Na concentration of 20 mM that combined with a ψ_b of 46 mV, would provide a $\Delta\tilde{\mu}_b^{Na}$ of 98 mV, more than four times greater than $\Delta\tilde{\mu}_b^{Cl}$. Thus the sum of $\Delta\tilde{\mu}_b^{Cl}$ and $\Delta\tilde{\mu}_b^{Na}$ would result in a net driving force for NaCl entry of 76 mV. Even if the intracellular Na activity were the same as that in the bathing media, the electrical potential difference across the basolateral membrane of 46 mV would be sufficient to energize Cl entry into the cell. These calculations illustrate the point that the electrochemical potential difference for Na is more than sufficient to energize the "uphill" movement of Cl into the cell. The Na gradient and negative intracellular voltage are ultimately maintained by the basolateral membrane Na-K-ATPase,[5] which is coupled to metabolic energy. These findings explain the inhibition of Cl secretion produced by inhibition of the Na pump with ouabain. Thus, no direct link between Cl entry and metabolic energy is required. A model that illustrates this mechanism of Cl entry is shown in Fig. 4.

After stimulation of Cl secretion, a_c^{Cl} was not altered (Table I). In the face of an increase in apical Cl conductance (7, 9) that allows an increased rate of Cl exit, the maintenance of a constant a_c^{Cl} indicates an equivalent increase in the rate of Cl entry into the cell across the basolateral membrane.[6] One possible explanation

[5] The basolateral membrane K permeability is an important factor determining the negative intracellular voltage (17). However, the K concentration gradient across the basolateral membrane is also dependent upon the activity of the Na-K-ATPase.

[6] An alternative explanation for the constancy of a_c^{Cl} is a situation in which Cl enters the cell via an electrically neutral Na-dependent mechanism and then is recycled back across the basolateral membrane by some other process under nonsecreting conditions. If stimulation of secretion inhibited the recycling of Cl, then Cl might exit across the apical membrane without a change in a_c^{Cl}. The absence of a basolateral Cl permeability (7) precludes an electrically conductive recycling but not a neutral process. It is also worth noting that such a recycling of Cl across the basolateral membrane under nonsecreting conditions would appear to be an inefficient use of metabolic energy.

for the enhancement of the rate of Cl entry is an increase in the electrochemical driving force for Cl entry. This explanation seems unlikely since a_c^{Cl} did not change and since $\Delta\tilde{\mu}_b^{Na}$ is more than sufficient to drive Cl entry against its gradient under both secreting and nonsecreting conditions. While an increase in $\Delta\tilde{\mu}_b^{Na}$ is possible if cAMP directly stimulates the Na-K-ATPase, thus lowering the intracellular Na activity, such an effect will probably not substantially alter $\Delta\tilde{\mu}_b^{Na}$. Evaluation of this possibility cannot currently be made since the effect of secretion rate on intracellular Na activity is unknown. A more likely explanation for the constancy of a_c^{Cl} is that some other factor regulates the rate of Cl entry. Although the identity of such a mediator is unknown, cAMP is a reasonable candidate to regulate the rate of Cl entry since the intracellular content of cAMP is known to increase with stimulation of secretion (2). Furthermore, there is a precedent for cAMP mediation of electrically neutral Cl entry mechanisms in isolated perfused shark rectal gland (25) and in NaCl absorption by flounder intestine (26). Thus, intracellular levels of cAMP may mediate both the rate of Cl exit (2, 7) and Cl entry in this secretory epithelium.

The loop diuretic, bumetanide, inhibited Cl secretion and decreased a_c^{Cl} providing direct evidence for an inhibition of Cl entry. This finding is consistent with the conclusion of a previous study (8), based on an equivalent electrical circuit analysis, that furosemide decreased the rate of Cl secretion by inhibiting Cl entry and decreasing the estimated a_c^{Cl}. This evidence that loop diuretics inhibit Cl secretion and a neutral Cl entry process in tracheal epithelium is in agreement with the suggestion that loop diuretics inhibit Cl cotransport processes in a variety of other epithelial (20) and nonepithelial cells (12).

These results, taken together, suggest that Cl entry is mediated by a NaCl cotransport process at the basolateral membrane and that the energy for Cl entry is derived from the transmembrane Na gradient. This situation is somewhat analogous to that found in epithelia performing coupled NaCl absorption such as gallbladder (15, 27) and intestinal epithelium (28) in which Cl entry into the cell across the apical membrane is directed against an electrochemical gradient. Thus, it appears that Cl secreting epithelia may share a common feature of transport with a variety of cells throughout the animal kingdom (29), i.e., Na-coupled transport that is dependent upon the transmembrane Na gradient.

Chloride exit at the apical membrane. These results indicate that Cl exit across the apical membrane occurs down a favorable electrochemical gradient, and thus, may be passive. The Cl permeability of the apical membrane varies, depending upon the presence or absence of secretagogues and the intracellular cAMP levels (2, 7). In the nonsecreting state there is minimal, if any, Cl conductance; following the addition of secretagogue the apical membrane is primarily Cl permeable. Since there is a favorable electrochemical gradient for Cl exit under both secreting and nonsecreting conditions, it is likely that the magnitude of the apical Cl permeability is an important factor controlling the rate of Cl exit and thus the rate of transepithelial Cl secretion.

While the electrochemical gradient for Cl across the apical membrane favors Cl exit, it is appropriate to ask whether the gradient is sufficient to account for the observed rate of secretion. The $\Delta\tilde{\mu}_a^{Cl}$ required for secretion will depend upon the specific mechanism of transport, which is unknown. However, as a starting point, I will assume a diffusional exit step so that an estimate of the membrane conductance required for secretion can be made. If the Cl current is 85 $\mu A \cdot cm^{-2}$ (Table I, 115 $\mu A \cdot cm^{-2}$ after stimulation minus 30 $\mu A \cdot cm^{-2}$ of Na absorption before stimulation) and the electrochemical driving force for Cl exit is 22 mV (Table I), then the Cl conductance required for diffusional Cl exit would be 3.9 $mS \cdot cm^{-2}$ (or a resistance of 260 $\Omega \cdot cm^{-2}$). This calculated value is in close agreement with an apical membrane conductance of ~4 $mS \cdot cm^{-2}$ determined from equivalent electrical circuit analysis of the epithelium (8, 9).[7] Thus, the data suggest that Cl exit may be entirely passive via an apical membrane transport process that need be no more complicated than diffusion.

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[7] The conductance of the apical membrane was estimated using an equivalent electrical circuit model in which there are two pathways for transepithelial current flow: a cellular pathway in parallel with a paracellular pathway. The cellular path consists of the two cell membranes in series, each modeled as an electrical resistance. The paracellular pathway is also represented as a resistance. Values for each of the circuit parameters can be obtained from intracellular electrophysiologic recordings obtained during the acute response to stimulation of Cl secretion (9). Under base-line nonsecreting conditions (indomethacin treated) apical membrane conductance is in the range of 0.4 to 0.5 $mS \cdot cm^{-2}$ (8, 9). Following stimulation of secretion the conductance of the apical membrane increases to values in the range of 3 to 5 $mS \cdot cm^{-2}$ (8, 9, 21). Although the apical membrane does contain a Na conductance, the magnitude of the Na conductance is much less than that of the Cl conductance under secreting conditions (the partial ionic conductance to Cl is ~20 times that for Na [9, 17]). Thus the total apical conductance primarily reflects the partial ionic conductance for Cl.

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