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Chloride Conductive and Cotransport Mechanisms in Cultures of Canine Tracheal Epithelial Cells Measured by an Entrapped Fluorescent Indicator

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Summary. To study Cl conductive and cotransport mechanisms, primary cultures of canine tracheal cells were grown to confluency on thin glass cover slips and on porous filters. Transepithelial resistance was >100 $\Omega \cdot cm^2$, and short circuit current (I_{sc} = 2–20 $\mu A/cm^2$), representing active secretion of Cl, increased >threefold with addition of 10 μM isoproterenol to the serosal solution. Cells made transiently permeable in hypotonic solution were loaded with the Cl-sensitive fluorophore 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) (5 mM, 4 min, 150 mOsm). The electrical properties of the cell monolayers were not altered by the loading procedure. Intracellular SPQ fluorescence was monitored continuously by epifluorescence microscopy (excitation 360 ± 5 nm, emission >410 nm). SPQ leakage from the cells was <10% in 60 min at 37°C. Intracellular calibration of SPQ fluorescence *vs.* [Cl] (0–90 mM) was carried out using high-K buffers containing the ionophores nigericin (5 μM) and tributyltin (10 μM); SPQ fluorescence was quenched with a Stern-Volmer constant of 13 M^{-1}. Intracellular Cl activity was 43 ± 4 mM. Cl flux was measured in response to addition and removal of 114 mM Cl from the bathing solution. Addition of 10 μM isoproterenol increased Cl efflux from 0.10 to 0.27 mM/sec. The increase was inhibited by the Cl-channel blocker diphenylamine-2-carboxylic acid (1 mM). In the absence of isoproterenol, removal of external Na or addition of 0.5 mM furosemide, reduced Cl influx by >fourfold. In ouabain-treated monolayers, removal of external K in the presence of 5 mM barium diminished Cl influx by >twofold, suggesting that Cl entry is in part K dependent. These results establish an accurate optical method for the real-time measurement of intracellular Cl activity in tracheal cells that does not require an electrically tight cell monolayer. The data demonstrate the presence of an isoproterenol-regulated Cl channel and a furosemide-sensitive cation-coupled transport mechanism.

Key Words fluorescence · SPQ · cell culture · chloride transport · trachea · isoproterenol · furosemide · Na-K-Cl transport

Introduction

Chloride secretion across the tracheal epithelium involves electrically neutral cotransport of Cl with Na and possibly K across the basolateral membrane followed by conductive movement of Cl across apical membrane Cl channels (Welsh, 1987). The apical membrane Cl channel is regulated primarily by a cAMP-dependent pathway (Smith et al., 1982); however, there are important influences of calcium and protein kinase C pathways (Frizzell, Rechkemmer & Shoemaker, 1986; Widdicombe, 1986; Barthelson, Jacoby & Widdicombe, 1987; Welsh, 1987). It is the regulation of this Cl channel that appears to be a central defect in the genetic disease, cystic fibrosis (Frizzell, 1987).

Monolayers of canine tracheal epithelial cells grown in primary culture have been used to examine tracheal Cl transport by short-circuit current, patch-clamp, microelectrode and tracer Cl methods (Welsh, 1986; Welsh & Liedtke, 1986; Widdicombe et al., 1987; Fong & Widdicombe, 1989). We have developed a quantitative fluorescence method to examine Cl transport in cultured tracheal epithelial cells based on the fluorescence of an entrapped Cl-sensitive fluorescent indicator. The method was validated by comparison of results with those from short-circuit current studies. Using this new methodology, we identified an isoproterenol-stimulated Cl conductance that was inhibited by diphenylamine-2-carboxylate, and a Na- and K-sensitive Cl cotransport mechanism that was inhibited by furosemide. The fluorescence methodology is technically easier than microelectrode methods which give similar information. In addition, unlike short-circuit current studies, the fluorescence method gives direct information about intracellular Cl activity and does not require that the cells form electrically tight junctions.

Materials and Methods

Materials

SPQ was synthesized as described previously (Krapf, Berry & Verkman, 1988*b*) and recrystallized three times from 1:1 methanol: H_2O. SPQ gave a single spot on reverse phase thin

Table 1. Composition of solutions

Component	Solutions									
	1	2	3	4	5	6	7	8	9	10
Na^+	130	25	25	101	101	0	0	101	101	101
K^+	5	120	120	5	5	5	5	5	0	0
Ca^{2+}	2	8	2	5	2	2	2	7	7	2
Ba^{2+}	0	0	0	0	0	0	0	0	5	5
$Choline^+$	0	0	0	0	0	0	101	0	0	0
Cl^-	114	0	153	0	114	0	114	0	0	114
$Gluconate^-$	29	165	0	0	0	0	0	106	101	0
$Isethionate^-$	0	0	0	106	0	5	0	0	0	0
$Acetate^-$	0	0	0	14	0	8	0	18	28	5
Mannitol	0	50	50	50	50	252	50	50	50	50

In addition, all solutions contained (in mM): Mg^{2+} 2, D-glucose 5; and HEPES/Tris 5. Solution pH was adjusted to 7.4.

layer chromatography (1 : 35 chloroform : methanol). Diphenylamine-2-carboxylate (DPC) was purchased from Aldrich (Milwaukee, WI) and added from a 100 mM ethanolic stock solution. All other chemicals were purchased from Sigma (St. Louis, MO). Solutions containing isoproterenol or furosemide were prepared freshly. The ionophores nigericin (K/H exchanger) and tributyltin (Cl/OH exchanger) were added from ethanolic stock solutions. The compositions of solutions used in these studies are given in Table 1.

Cell Culture

Primary cultures of canine tracheal epithelial cells were grown as described in detail elsewhere (Coleman, Tuet & Widdicombe, 1984). Briefly, strips of tracheal mucosa were digested with collagenase and the dispersed cells were plated at a density of 1.25×10^5 cells/cm^2. For fluorescence measurements, cells were plated on 18-mm diameter round glass coverslips. For short-circuit current (I_{sc}) measurements, cells were plated on porous Nuclepore filters. Both glass coverslips and Nuclepore filters were coated with 12 μg/cm^2 of human placental collagen. Cells were grown in a 50 : 50 mix of Eagles' Modified Medium and Hams F12 supplemented with 5% fetal bovine serum in 5% CO_2/95% air at 37°C. The culture medium was changed every 2–3 days. Experiments were performed after cells reached confluency in 14–28 days after plating. It was established previously that the electrical properties of these cells remain constant over this period. Every cell preparation was plated on both glass coverslips and Nuclepore filters. Fluorescence measurements were performed only after demonstrating a transepithelial resistance of $>100\ \Omega \cdot$ cm^2 and an increase in I_{sc} of $>5\ \mu$A/cm^2 in response to serosal addition of 10 μM isoproterenol.

Short-Circuit Current Measurements

Cell monolayers grown on Nuclepore filters were mounted in Ussing chambers with 0.5 cm^2 exposed area. The edges of the Lucite half-chambers were coated with Dow-Corning high-vacuum grease to minimize edge damage. Ussing chambers were connected to recirculating gas-lift oxygenators containing buffer bubbled with 100% O_2 at 37°C. Transepithelial potential difference (PD) and I_{sc} were measured as described previously (Coleman et al., 1984). Transepithelial resistance was determined from the size of 200-msec current pulses passed across short-circuited tissues to displace PD by +0.5 mV. For measurement of rapid changes in I_{sc} following Cl addition or removal, the gas-life oxygenators were disconnected and fluid was continuously perfused (40 ml/min) through both half-chambers (volume 0.75 ml) with a peristaltic pump.

To test whether the SPQ hypotonic loading procedure alters cell viability and Cl transport, electrical properties were examined in paired monolayers from three dogs. It was found that loading with SPQ by the procedure described in the next section was without effect on transepithelial resistance (R_t), baseline I_{sc} or the response in I_{sc} to isoproterenol (Table 2).

Fluorescence Measurements

Tracheal cells were loaded with SPQ by a transient permeabilization procedure (Chao et al., 1989). Cells were incubated in a 1 : 1 mixture of solution 1 and distilled H_2O containing 5 mM SPQ for 4 min at 23°C. Cells were then washed for 1 min with solution 1 before transfer to the perfusion chamber (volume 180 μl) in which cell fluorescence could be measured in response to rapid solution changes in an inverted epifluorescence microscope. The undersurface of the glass coverslip was the lower boundary of the cell chamber for contact with immersion objectives. Cultured cell monolayers were perfused with experimental solutions by a gravity-driven system at a rate of 15–20 ml/min.

SPQ fluorescence was measured using a Nikon inverted epifluorescence microscope (Diaphot, Japan). Fluorescence was excited at 360 nm by a 100-W tungsten-halogen lamp powered by a stabilized DC supply in series with an O.D. 2 neutral density filter, an UG1 black glass filter (Schott Glass, Duryea, PA) and a 360 ± 5 nm six-cavity interference filter (Omega Optical, Brattleboro, VT). Excitation light was reflected by a 400-nm fused silica dichroic mirror (Omega Optical) and illuminated the cells through a 40× glycerol immersion quartz objective (Leitz-Wezlar, Germany, N.A. 0.65, working distance 0.35 mm). Emitted light was filtered by a 410-nm low autofluorescence cut-on filter and detected by a Hamamatsu R928S photomultiplier (Middlesex, NJ) powered by an Ealing high voltage supply/amplifier

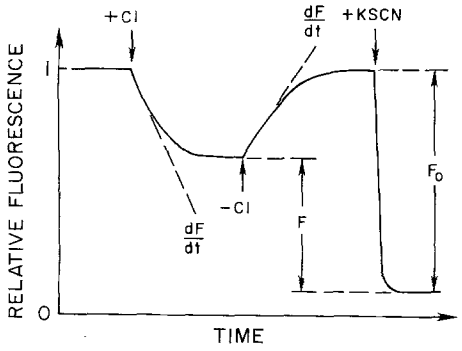

Fig. 1. Calculation of Cl flux from SPQ fluorescence data. A relative fluorescence of unity corresponds to an intracellular Cl activity of zero (*see* text for explanation)

(South Natick, MA). The amplified signal was digitized by an analogue-to-digital converter (Interactive Microware, College Point, PA) at a rate of 30 points/sec and averaged over 1-sec intervals by an IBM PC/AT computer. Concentric circular iris diaphragms were used to illuminate and measure ~50% of the field of vision (10–15 cells).

To check for uniformity of cell staining, cells were imaged using a 100× oil immersion objective (Nikon, fluorotar, N.A. 1.30, working distance 0.17 mm) by a SIT camera (DAGE-MTI, Michigan City, IN) interfaced to a PC/AT computer. In addition, a ratio-imaging technique was used to compare the distribution of SPQ with that of the pH indicator, BCECF. Single cells loaded with SPQ and with BCECF were imaged separately for SPQ and BCECF (excitation 480 nm, emission >515 nm) fluorescence. The camera was modified to operate at fixed gain for quantitative imaging.

Cl Flux Calculations

Cl influx and efflux rates (J_{Cl}, mM/sec) were calculated from the intracellular SPQ fluorescence time course as depicted in Fig. 1. J_{Cl} at time t_0 is related to the total SPQ fluorescence signal in the absence of Cl (F_0), the SPQ fluorescence signal at time t_0 (F) and the fluorescence *vs.* time slope at time t_0 (dF/dt) by the relation (Chen et al., 1988),

$$J_{Cl} = \frac{F_0}{K_{Cl}F^2}\frac{dF}{dt} \quad (1)$$

where K_{Cl} is the Stern-Volmer constant for quenching of intracellular SPQ by Cl. In the experimental curves, dF/dt was determined from the derivative of a single exponential function fitted to the data curve in the region of t_0. F_0 was determined from the difference in fluorescence signal measured in cells in zero Cl buffer and after KSCN addition which quenched all SPQ fluorescence (Illsley & Verkman, 1987). K_{Cl} was determined by an ionophore calibration procedure (*see* Results). In the presence of slow leakage of SPQ from cells (generally <10%/hr), F_0 was interpolated for fluorescence levels at zero Cl measured at the start of the experiment and just prior to KSCN addition. The fluorescence level after KSCN addition was obtained from the infinite time point of a single exponential function fitted to the time course of decreasing fluorescence after KSCN addition.

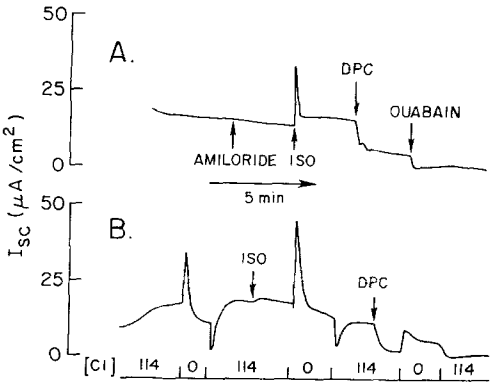

Fig. 2. Short-circuit current response of tracheal cultures. (*A*) Stimulation of Cl secretion by isoproterenol. Addition of amiloride (10^{-5} M) to the mucosal bath caused a small decline in I_{sc} due to abolition of Na absorption. Addition of isoproterenol to the serosal solution (10^{-5} M) stimulated I_{sc} rapidly; the increased I_{sc} was inhibited by mucosal application of the Cl channel blocker, DPC (10^{-3} M). Addition of ouabain to the serosal solution (10^{-4} M) abolished the remaining I_{sc}. (*B*) Effects of Cl removal from and readdition to both the mucosal and serosal solutions. Isoproterenol (10^{-5} M) and DPC (10^{-3} M) were added to the serosal or mucosal solutions, respectively, where indicated. Amiloride (10^{-5} M) was present in the mucosal solution throughout the experiment

This value did not differ significantly from the fluorescence of a cell monolayer that had not been loaded with SPQ.

Results

Short-Circuit Current Measurements

All cultures had a transepithelial resistance $R_t > 100\ \Omega \cdot cm^2$ with an average of 201 ± 24 $\Omega \cdot cm^2$. Baseline I_{sc} varied from 2–20 $\mu A/cm^2$ (mean ± SE, 6.7 ± 0.9 $\mu A/cm^2$, $n = 11$ cultures). A standard protocol, illustrated in Fig. 2*A*, was used to test the ability of isoproterenol to stimulate Cl secretion. First, Na absorption was inhibited by amiloride. Isoproterenol was then added, followed by the Cl channel blocker, DPC (Wangemann et al., 1986). Finally, residual I_{sc} was abolished with ouabain. As reported previously (Widdicombe et al., 1987), all cultures showed a biphasic response to isoproterenol; I_{sc} increased to 2–4× the baseline value within 15 sec and then decreased to a steady level between the baseline and maximum value. The responses to isoproterenol showed no tachyphylaxis. Isoproterenol could be washed out and readded up to nine times in a 2-hr period with no diminution in response (*data not shown*).

To compare Cl transients measured by the short-circuit current technique with those measured by SPQ fluorescence, rapid changes in I_{sc} were measured following Cl removal from and addition to the

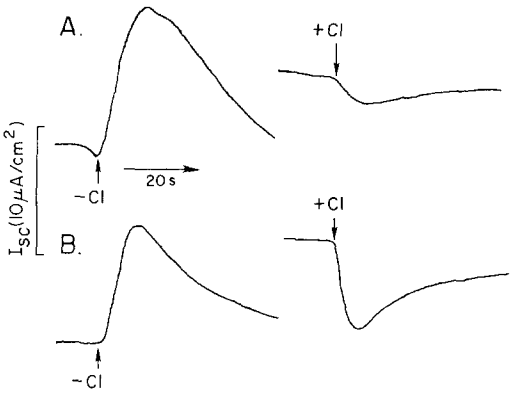

Fig. 3. Effects of bumetanide on response of I_{sc} to Cl removal and readdition. (*A*) Cl removal (left) and readdition (right) in the absence of bumetanide. (*B*) The same maneuvers following 15-min exposure to bumetanide (10^{-4} M on both sides). Similar results were obtained on four other tissues

mucosal and serosal solutions done first without isoproterenol, then with isoproterenol and then with isoproterenol and DPC (Fig. 2*B*). Upon Cl removal, there was a rapid increase in I_{sc}, followed by a slower decrease toward zero over ~2 min. The transient increases in I_{sc} were due primarily to the movement of Cl across the cell apical membrane, with maintenance of electroneutrality by an equivalent movement of K across K-selective channels in the basolateral membrane (Welsh, 1983*a*). The subsequent decrease was due to the time course of cell Cl depletion. I_{sc} failed to reach zero because of the presence of amiloride-insensitive Na absorption (Widdicombe & Barthelson, 1988). A transient decrease in I_{sc} was seen upon addition of Cl. The amplitude of these transients was increased by isoproterenol, and reduced by DPC. In results from seven cell cultures, isoproterenol increased the amplitude of the upward transients in I_{sc} from 17 ± 2 to 33 ± 6 $\mu A/cm^2$ ($P < 0.05$, $n = 15$). In the presence of isoproterenol, DPC reduced the amplitude of the transient from 38 ± 8 to 6 ± 1 $\mu A/cm^2$ ($P < 0.05$, $n = 11$).

It was noted that the area under the transient increases in I_{sc} was consistently greater than the area under the transient decreases in I_{sc} (Fig. 3*A*). This probably occurs because the electrically neutral operation of the basolateral Na/K/2Cl carrier contributes differentially to the entry and exit of K and Cl. Support for this idea is provided by the finding that inhibition of the cotransporter with bumetanide increases the size of the transient caused by Cl addition, so that it becomes similar to the size of the transient caused by Cl removal (Fig. 3*B*).

The area under the transient caused by Cl removal (the time integral of current) gives the total Cl and K lost from the cells (in coulombs), and can be used to estimate the intracellular Cl activity. Total I_{sc} at any time will be the sum of transients due to ion loss from the cells and two net transepithelial transport processes: Cl secretion and amiloride-insensitive Na absorption (Widdicombe & Barthelson, 1988). On Cl removal, net Cl secretion will be abolished instantaneously, and I_{sc} will equal the sum of Na absorption and cellular loss of K and Cl. Upon complete depletion of intracellular Cl, vectorial Na absorption will be the only contributor to I_{sc}. Therefore, in estimating the loss of KCl during the upward transients, the perimeter of the transient was defined by a vertical line from the time of Cl removal and a horizontal line from the steady-state level of I_{sc} in the absence of Cl. In Fig. 2*B*, the area of the I_{sc} transient in the presence of isoproterenol was 960 $\mu C \cdot cm^{-2}$ (10 $nEq \cdot cm^{-2}$). Our cells are ~5 μm in height (Widdicombe et al., 1987) corresponding to a volume of 0.5 $\mu l \cdot cm^{-2}$, about half of which (0.25 $\mu l \cdot cm^{-2}$) will be intracellular fluid. Dividing the Cl content by the water content yields a $[Cl]_i$ of 40 mM. The close correspondence between $[Cl]_i$ estimated in this way, and $[Cl]_i$ determined with ion-sensitive microelectrodes (Shorofsky, Field & Fozzard, 1984) supports our hypothesis that the transients represent conductive loss of Cl (and K) from the cells.

Fluorescence Measurements

Cell SPQ Loading and Imaging

Cells were loaded with SPQ by a hypotonic loading procedure as described in Materials and Methods. Typical images of SPQ-loaded cells are shown in Fig. 4. The cell fluorescence was distinctly demarcated and appeared to be uniform within individual cells. There were no bright spots due to SPQ binding to cell components or precipitation in the intercellular spaces. Intracellular distribution of an ion-sensitive indicator is an important concern for quantitative photometry measurements. A ratio-imaging technique, which is not sensitive to monolayer thickness, was used to compare the distribution of SPQ with that of the extensively used pH indicator, BCECF, which was assumed to reside only in the cytoplasmic compartment. Ratio images of tracheal cells loaded with SPQ and with the fluid-phase pH indicator BCECF were found to be highly uniform without distinct variations across the cell. The variation in ratio values across the cell was under 5%. SPQ leaked out of loaded cells <10% per hr at 37°C, similar to results found in cultured renal cells and fibroblasts (Chao et al., 1989).

The effect of SPQ hypotonic loading on electrical properties of the tissue monolayers was ex-

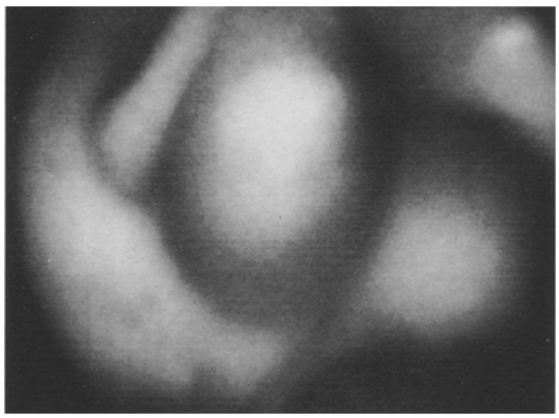

Fig. 4. Fluorescence micrograph of primary cultures of canine tracheal epithelial cells loaded with SPQ. Cell were loaded with SPQ, perfused with a Cl-free buffer (solution 4) and imaged as described in Materials and Methods. Background signal was subtracted digitally. Final magnification is 1,250×

amined. SPQ loading did not alter R_t, baseline I_{sc}, and the increase of I_{sc} in response to isoproterenol (Table 2).

Calibration of Intracellular SPQ Fluorescence

To measure transients in intracellular Cl, it was necessary to establish the relationship between intracellular SPQ fluorescence and Cl activity. Because neither the fluorescence excitation nor emission spectra of SPQ have an isosbestic wavelength, a two point calibration scheme was required in every experiment in which SPQ fluorescence was measured in the absence of Cl and in the presence of a solution which quenched all SPQ fluorescence (KSCN + valinomycin, *see above*). SPQ fluorescence was calibrated against intracellular [Cl] using a double ionophore procedure. Intracellular [Cl] was set equal to external [Cl] by perfusing calibration solutions containing high K (120 mM), the K/H exchange ionophore nigericin and the Cl/OH exchange ionophore tributyltin (Krapf et al., 1988*a*).

Figure 5 (top) shows a record of a calibration carried out in the Cl concentration range from 0 to 90 mM. Cell fluorescence signals changed reversibly in response to changes in external solution [Cl]. The fluorescence signal decreased at a rate of ~15% per hr due to SPQ leakage; the relatively fast rate of SPQ leakage in the presence of nigericin/tributyltin has been noted in other cells and is believed to be due to cell toxicity. Figure 5 (bottom) shows a Stern-Volmer plot for quenching of intracellular SPQ fluorescence by Cl determined from the calibration data. The Stern-Volmer constant was 13 M^{-1}. This value is similar to that determined for

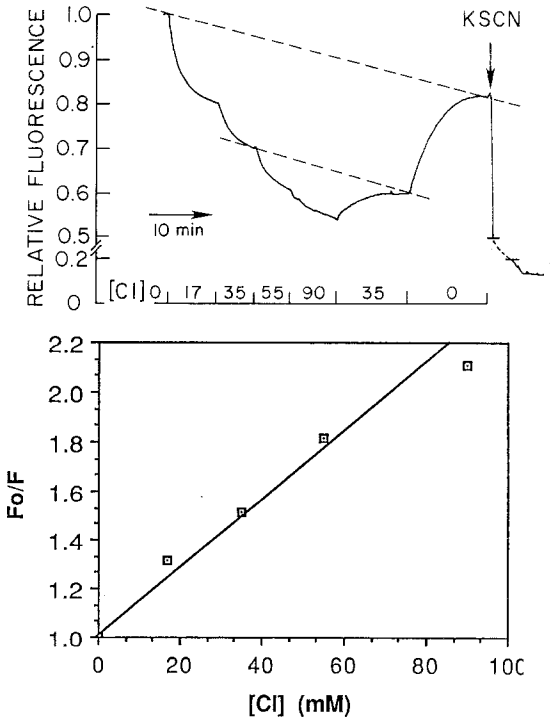

Fig. 5. Intracellular calibration of SPQ. *Top:* Monolayers of primary cultures of canine tracheal cells were perfused at room temperature with solutions containing the ionophores nigericin (5 μM) and tributyltin (10 μM). Calibration solutions were prepared by mixing solutions 2 and 3. Dashed lines represent loss of SPQ fluorescence due to leakage. *Bottom:* Calibration curve in the form of a Stern-Volmer plot, where F_0/F is SPQ fluorescence in the absence of Cl divided by that in the presence of Cl. The fitted Stern-Volmer constant was 13 M^{-1}

Table 2. Effect of SPQ loading on electrical properties of dog tracheal cells

	Control	SPQ
R_t ($\Omega \cdot cm^2$)	142 ± 20	164 ± 22
I_{sc} ($\mu A/cm^2$)	10 ± 1	9 ± 1
ΔI_{sc} by iso ($\mu A/cm^2$)	8 ± 3	9 ± 4

R_t, I_{sc} and the increase in I_{sc} induced by addition of isoproterenol were examined in paired tissues with and without SPQ hypotonic loading. Values are given as mean ± SEM for experiments performed in six monolayers from three dogs.

SPQ in LLC-PK1 cells and 3T3 Swiss fibroblasts (13 M^{-1}, Chao et al., 1989) and in cells of the intact kidney proximal tubule (12 M^{-1}, Krapf et al., 1988*a*).

Isoproterenol-Stimulated Cl Conductive Pathway

Electrophysiological studies have identified a Cl conductive pathway at the apical membrane of ca-

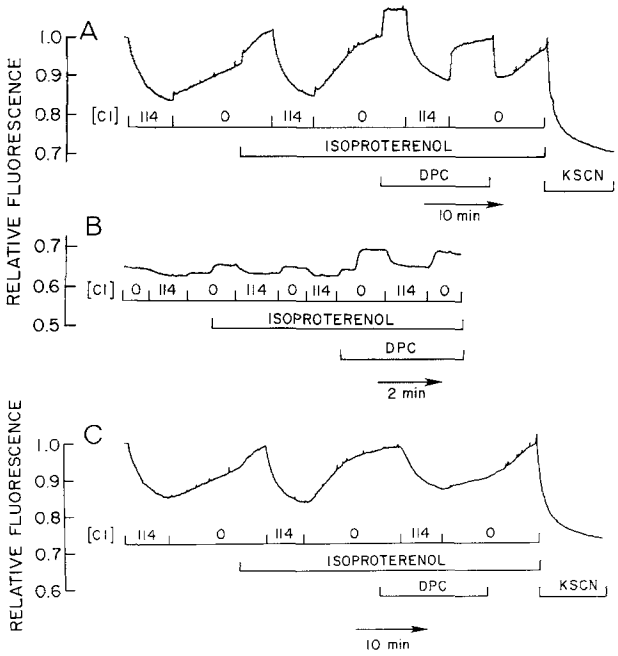

Fig. 6. Effect of isoproterenol on Cl flux. The experiment was carried out at 37°C. (*A*) Cl (114 mM) was added into and removed from the bath by changes between solutions 4 and 5 in the presence and absence of 10 μM isoproterenol. In the third cycle of external Cl addition/removal, 1 mM DPC was present. (*B*) The same protocol was performed using a coverslip that did not contain cells to determine the Cl-independent signal changes due to solution components. (*C*) Curve *A* was corrected by subtracting the optical artifacts determined in *B*. Optical artifacts were corrected in subsequent figures

Table 3. Isoproterenol-stimulated Cl flux

	Influx	Efflux
$n = 5$		
Control	0.21 ± 0.06	0.09 ± 0.01
+ Isoproterenol	0.43 ± 0.11	0.44 ± 0.06
Paired ratio [(iso/control)]	2.19 ± 0.29[a]	5.25 ± 1.15[a]
$n = 3$		
+ Isoproterenol	0.29 ± 0.03	0.23 ± 0.05
+ Iso + DPC	0.11 ± 0.03	0.07 ± 0.02
Paired ratio [(iso + DPC)/iso]	0.37 ± 0.06[a]	0.34 ± 0.09[a]

Initial rates of Cl influx following Cl addition and efflux following Cl removal are given as mean ± SEM in units of mM/sec. For experimental protocol, refer to legend to Fig. 6. The paired ratio represents the mean ± SEM for the ratio indicated in brackets. Individual paired ratios were calculated from measurements performed on a single cell monolayer.

[a] Denotes significance ($P < 0.05$) of statistical comparisons between the paired ratio and unity.

nine tracheal epithelia, which is activated by beta-adrenergic agonists which increase intracellular cAMP (Welsh, 1986, 1987). To study this mechanism by use of intracellular SPQ fluorescence, transmembrane Cl flux rates were measured in response to Cl addition to and removal from the bath in the absence and presence of isoproterenol (10 μM). Cell monolayers were first perfused with a Cl-free buffer (solution 4). To induce Cl fluxes, 114 mM Cl was added into and washed out from the bathing medium repeatedly by altering perfusion fluid between solution 4 (containing no Cl) and solution 5 (containing 114 mM Cl). Figure 6*A* shows an experiment performed at 37°C. Abrupt displacements in the fluorescence signal which occur within the mixing time of the perfusion chamber were observed when solutions were changed. These sudden changes in fluorescence level were optical artifacts of light scattering or dim fluorescence from solution components. They could be reproduced by changing perfusion solutions in the absence of the cell monolayers (Fig. 6*B*). Figure 6*C* shows results of the same experiment after correction for these optical artifacts. Addition of isoproterenol promptly increased the rate of Cl efflux. Six to 8 min after Cl (114 mM) was added into Cl-free bathing medium, intracellular Cl concentration was elevated to 52 mM, equivalent to 39 mM in Cl activity assuming that the Cl activity coefficient in cytosol is 0.75. This value is comparable to that estimated from short-circuit current studies and to that measured previously in native canine tracheal epithelial cells using Cl-selective microelectrodes (47 mM, Shorofsky et al., 1984; 37 mM, Welsh, 1983*b*). Following addition of isoproterenol, the initial Cl efflux rate was increased by approximately threefold. The stimulation by isoproterenol was abolished by addition of the Cl channel blocker diphenylamine-2-carboxylate (DPC). Data from a series of measurements on separate monolayers are summarized in Table 3.

The ability of isoproterenol to stimulate Cl flux indicates that isoproterenol has access to the basolateral plasma membrane. In addition, the ability to examine the characteristics of a furosemide-sensitive Na/K/2Cl cotransporter (*see below*) indicates that ions and some inhibitors have access to the basal membrane, probably by passing across the paracellular junctions. Because measured Cl flux is the sum of at least two components, conductive and cotransport, we reasoned that it should be possible to measure a greater fractional stimulation of conductive Cl flux by removal of Na to inhibit Na-dependent Cl cotransport. As shown in Fig. 7, in the absence of solution Na, isoproterenol remarkably increased the initial Cl efflux rate.

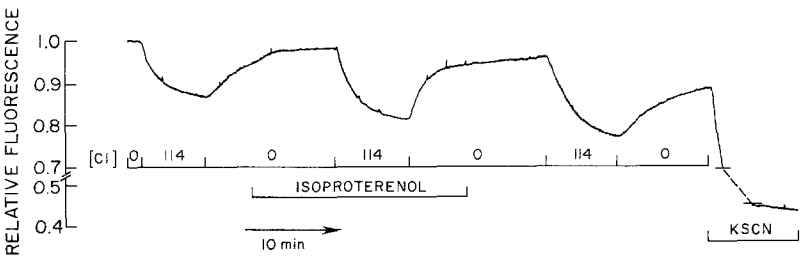

Fig. 7. Effect of isoproterenol on Cl flux in the absence of Na at 23°C. Cl was added to and removed from the external solution in the absence of Na using solutions 6 and 7. Where indicated, 10 μM isoproterenol was present

Table 4. Cation-coupled Cl cotransport

	Cl influx	Cl efflux
Furosemide		
$n = 4$		
Control	0.28 ± 0.07	0.32 ± 0.10
+ 0.5 mM Furosemide	0.09 ± 0.04	0.06 ± 0.03
Paired ratio	0.29 ± 0.09[a]	0.21 ± 0.10[a]
Na removal		
$n = 3$		
Control	0.29 ± 0.06	0.34 ± 0.13
− Na	0.03 ± 0.01	0.05 ± 0.02
Paired ratio	0.11 ± 0.03[a]	0.16 ± 0.02[a]
K removal		
$n = 4$		
Control	0.20 ± 0.06	0.23 ± 0.10
− K	0.09 ± 0.04	0.15 ± 0.10
Paired ratio	0.45 ± 0.13[a]	0.65 ± 0.12[a]

Initial rates of Cl influx following Cl addition and efflux following Cl removal are given as mean ± SEM in units of mM/sec. The experimental protocol is given in the legend to Fig. 8.
[a] Denotes significance ($P < 0.05$) of statistical comparisons between paired ratio and unity.

Cation-Coupled Cl Cotransport: Inhibition By Furosemide

Loop diuretics including furosemide and bumetanide strongly inhibit cation-coupled Cl cotransport (Geck & Heinz, 1986). Short-circuit current (I_{sc}) measurements and ^{36}Cl tracer flux studies have shown that furosemide is a potent inhibitor of Cl secretion in canine tracheal epithelia (Widdicombe, Nathanson & Highland, 1983). The inhibitory effect of furosemide on Cl transport was examined to estimate the fraction of total Cl flux occurring via a cotransport mechanism. Typical experimental findings are given in Fig. 8*A*. Following the first cycle of external Cl addition/removal, 0.5 mM furosemide was added; another Cl addition/removal cycle was carried out to induce transmembrane Cl fluxes. Furosemide reduced the initial Cl influx rate by 70–80%. The results are summarized in Table 4. These results show that in the absence of isoproterenol, the majority of Cl influx in response to gradients of Cl is via a furosemide-sensitive pathway.

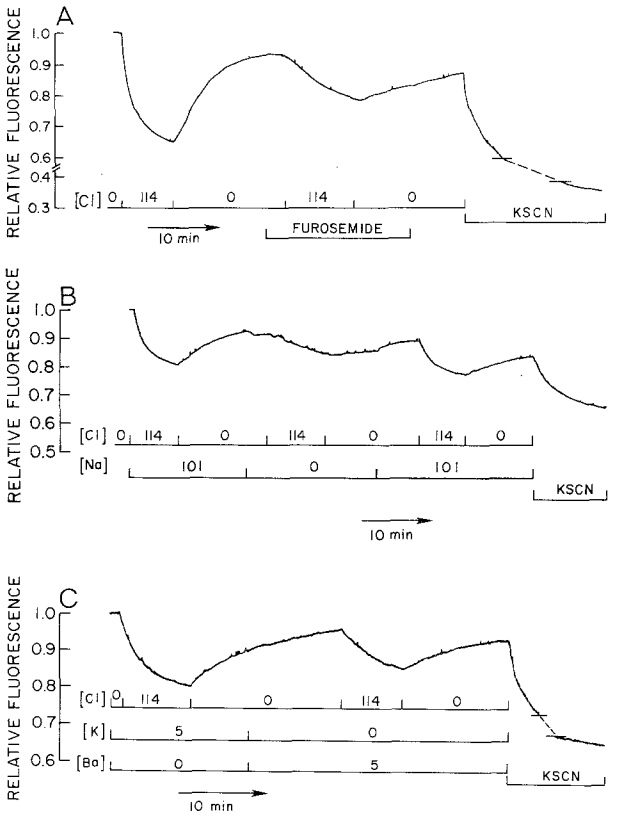

Fig. 8. Furosemide-sensitive, cation-coupled Cl cotransport in tracheal cells at 23°C. Results from a series of monolayers are summarized in Table 4. (*A*) Effect of 0.5 mM furosemide addition. External solutions were switched between solutions 4 and 5 in the absence and presence of 0.5 mM furosemide. (*B*) Effects of Na removal. External solutions were switched between solutions 4 and 5 (Na present) and 6 and 7 (Na absent). (*C*) Effects of K removal in the presence of barium and ouabain. Cells were treated with 0.1 mM ouabain for 45 min prior to experiments. Solutions were changed from 8 and 5 (K present) to 9 and 10 (K absent)

Cation-Coupled Cl Cotransport: Na Dependence

The effect of Na removal on Cl flux was examined. Transmembrane Cl fluxes were induced by addition and removal of 114 mM Cl in the presence and absence of external Na (Fig. 8*B*). The initial rate of Cl

influx was decreased reversibly by ~85% by Na removal (Table 4). Because 70–80% of Cl flux was furosemide-sensitive, the inhibitory effect of Na removal supports the presence of a furosemide-sensitive Na-dependent Cl cotransport mechanism. Na removal might also have a small effect on conductive Cl entry by lowering the total plasma membrane cation conductance required to minimize Cl-gradient-induced membrane hyperpolarization. It is predicted that this effect should be small because of the high plasma membrane K conductance and the small fraction of total Cl flux that is conductive in the absence of isoproterenol.

Cation-Coupled Cl Cotransport: Is Cl Entry Dependent Upon External Potassium?

Electroneutral Na-K-2Cl cotransport has been identified in a variety of cell types as reviewed by Geck and Heinz (1986). However, the role of K in regulating basolateral Cl entry in tracheal epithelial cells has remained controversial (Welsh, 1987). It was shown previously (Greger & Schlatter, 1981) that Na-K-2Cl cotransport can be inhibited effectively by omitting external K and adding the K-channel blocker barium. Because K removal from the bathing media will inhibit Na-K pump activity and therefore reduce the basolateral Na gradient, the major driving force for basolateral Cl uptake, cell monolayers were pretreated with ouabain (0.1 mM) for 45 min before experiments. This strategy has been used to examine other carrier-mediated ion transport systems (Weinman & Reuss, 1984). As shown in Fig. 8*C*, the initial rate of Cl influx induced by external Cl addition was diminished significantly in the absence of K and in the presence of 5 mM barium. In experiments performed in four monolayers, initial Cl influx rate was reduced by 55% (Table 4). The effect of furosemide on Cl flux in the absence of K was also examined. In two experiments carried out in monolayers pretreated with ouabain and perfused with K-free solution containing Ba, the initial rate Cl influx rate was further reduced by ~50% with addition of 0.5 mM furosemide. These results indicate that Na-dependent Cl transport is significantly but not completely dependent upon K under the conditions of our experiments.

Discussion

The major goals of this study were: (*i*) to establish a rapid optical method to measure transients of intracellular [Cl] optically in tracheal epithelial cells, and (*ii*) to apply this methodology to examine Cl conductive and cotransport mechanisms. The fluorescence method has a number of advantages over Cl-sensitive microelectrodes for measurement of intracellular Cl activity. First, the fluorescence method does not cause direct cell membrane damage as do ion-selective microelectrodes. Ussing chamber experiments showed that viability of the cell monolayers was not affected by the SPQ loading procedure. Additionally, the fluorescence method is technically relatively simple, and can be used to measure Cl transients in one or many cells simultaneously that do not form electrically tight junctions. The highly polar Cl-sensitive fluorophore SPQ was loaded into cells made transiently permeable by a hypotonic loading procedure which has been shown to be effective in a variety of cell types (Chao et al., 1989). The fluorophore SPQ has been used to study Cl transport in several membrane vesicle systems (Chen & Verkman, 1988; Chen, Illsley & Verkman, 1988; Pearce & Verkman, 1989) including tracheal apical vesicles (Fong et al., 1988) and in phospholipid vesicles (Verkman et al., 1989). It has been used in intact epithelial cells of the kidney proximal convoluted tubule (Krapf et al., 1988*a*); however, rapid leakage rates (50% leakage in 8 min at 37°C) limited its practical use. Cell cultures grown to monolayers are good preparations for optical measurement of membrane Cl transport because of their geometry, homogeneity of the cell types and generally better availability than the original tissue.

Short-Circuit Current Measurements

Short-circuit current measurements were performed routinely as a screening procedure to check the quality of the cell monolayers. Fluorescence measurements were performed only when the monolayers were electrically tight and showed stimulation of short-circuit current by isoproterenol. Approximately 90% of cultured monolayers fulfilled these criteria when tested more than 10 days after plating. A novel variation of the short-circuit current approach was developed in which transients in short-circuit current were recorded in response to rapid Cl removal from or addition to bathing solutions. By assessing the total Cl loss/uptake following cell Cl loading/unloading, intracellular Cl activity could be estimated. The rapid transient increase in short-circuit current, which occurred within the mixing time, was interpreted in terms of the instantaneous Cl current through the cell apical membrane in response to the Cl gradient. The slower time course of short-circuit current following the initial transient caused by addition or removal of external Cl was interpreted in terms of changes in $[Cl]_i$. Importantly, the time course of decay of the

I_{sc} transients produced by Cl removal was very similar to the time course of increase in SPQ fluorescence produced by the same maneuver, suggesting that both techniques are indeed measuring changes in $[Cl]_i$.

Fluorescence Studies of Tracheal Cl Transport

A paired analysis was used in all experiments because of the variability in Cl flux rates. The variability may be in part related to the time-dependent changes in electrophysiological properties of the cell cultures. It has been observed that apical membrane potential of primary cultures of dog tracheal cells declines gradually 1–2 weeks after plating, together with a slight decrease in transepithelial resistance (Welsh, 1985). In addition, there is a considerable biological variability of primary cell cultures in general. As shown in previous studies (Widdicombe et al., 1987) and by the absence of vasopressin- and thrombin-induced elevations in intracellular calcium (*unpublished results*), the dog tracheal cells used in these experiments do not have detectable contamination by fibroblasts.

As expected, an increase in Cl transport in response to isoproterenol was demonstrated. The increase was inhibited by DPC, indicating the existence of an isoproterenol-stimulated Cl conductive pathway. In the absence of external Na, the stimulating effect of isoproterenol on Cl efflux was enhanced due to inhibition of cation-coupled Cl transport. These results show that the fluorescence method is a usable screening procedure for the presence of hormonally regulated Cl transport in cultured cells, such as those from patients with cystic fibrosis, in which cAMP-regulated Cl conductance is abnormal (Welsh & Liedtke, 1986; Frizzell, 1987). In addition, the fluorescence method can be used to screen cells that lack tight junctions and have low transepithelial resistance, such as cystic fibrosis epithelial cells that have been transformed by the SV40 virus (Gruenert et al., 1988).

In the absence of isoproterenol, the majority of Cl influx in response to Cl gradients was furosemide sensitive. Na removal (in the absence of furosemide) also inhibited the majority of Cl influx, indicating that the furosemide-sensitive Cl flux required Na. The role of K in mediating the basolateral transport of Cl in tracheal epithelia has been controversial. It was found in dog tracheal epithelium that the oxygen cost for Cl transport was significantly less than that required for Na transport (Welsh, 1984). However, oxygen consumption studies in bovine trachea suggested that the metabolic costs for Na and Cl transport were about equal (Durand, Durand-Arczynska & Shoenenweid, 1986). In isolated dispersed tracheal epithelial cells, no K-dependence of Na and Cl influx was seen (Widdicombe et al., 1983). In the mouse medullary thick ascending limb, it was reported recently that the K requirement for NaCl symport may depend upon hormonal presence (vasopressin) and cell volume (Sun & Hebert, 1989).

Under the conditions of our experiments performed in ouabain-treated tissues, both Na-Cl and Na-K-2Cl cotransport modes appear to be present. Ouabain treatment slowed the initial Cl influx rates because of dissipation of the Na gradient (Weinman & Reuss, 1984) which was important for Na-dependent Cl uptake. The initial Cl influx rate was reduced further by K removal in the presence of barium. To eliminate any K effect from the serosal side of the epithelium, addition of Ba was important to block K back-leak from the cell into the "unstirred" layer near the basolateral membrane. This was important in our experiments, where the submucosal compartment, enclosed by the basolateral membrane and glass coverslip, was poorly perfused. Addition of barium may depolarize the apical and basolateral membrane potential. This should facilitate the conductive Cl entry upon cell Cl loading. The greatly reduced rate of the initial Cl influx resulted from K removal, in the presence of Ba, suggests that K plays a role in mediating the Na-dependent Cl uptake. However, K removal did not inhibit Na-K-2Cl cotransport completely. We conclude that, under our experimental conditions, NaCl symport is, at least partly, K dependent. Further experiments are required to define possible dependences of hormones or cell volume on the K requirement.

In conclusion, we have established methodology to measure real-time intracellular Cl transients optically and to investigate cellular mechanisms for Cl transport in primary cultures of canine tracheal cell monolayers. The response of cell Cl permeability to isoproterenol and the time course of intracellular Cl transients measured by SPQ fluorescence were in agreement with short-circuit current measurements. Isoproterenol-stimulated Cl conductance and Na-dependent furosemide-inhibited Cl transport pathways were clearly demonstrated. Under the conditions of our experiments, both Na-Cl and Na-K-2Cl cotransports are present.

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