Cystic fibrosis decreases the apical membrane chloride permeability of monolayers cultured from cells of tracheal epithelium

(CI secretion/Na absorption/Using chambers/intracellular microelectrodes)

J. H. Widdicombe*, M. J. Welsh†, and W. E. Finkreiner*

*Cardiovascular Research Institute, and the Departments of Physiology and Pathology, University of California, San Francisco, CA 94143; †Laboratory of Epithelial Transport, and the Pulmonary Division, Department of Internal Medicine, University of Iowa Hospitals, Iowa City, IA 52242

Communicated by John A. Clements, May 28, 1985

ABSTRACT The tracheal mucosa from a 12-year-old girl was digested with collagenase 4 hr after her death from cystic fibrosis. Forty million viable cells were obtained. The cells, plated at 10⁶ per cm² onto four Nuclepore filters coated with human placental collagen, formed confluent monolayers after 1 day. Their ultrastructure was similar to that of normal human cells. They were studied in conventional Using chambers or with intracellular microelectrodes on days 5–7 after plating. The monolayers displayed a resistance of 380 ± 50 Ω cm² and short-circuit current (Isc) of 1.8 ± 0.4 μA cm⁻². This resistance is similar to that obtained for dog or normal human monolayers. The Isc is less than normal human (∼3 μA cm⁻²) or dog (∼10 μA cm⁻²) cells. The cystic fibrosis cells resembled normal monolayers in that serosal ouabain and mucosal amiloride inhibited Isc, while mucosal ouabain or serosal amiloride had no effect. They differed from normal human or dog cells in that Isc was not inhibited by bumetanide and the stimulation of Isc by amiloride or prostaglandin E₂ was greatly reduced or abolished. Addition of isoproterenol depolarized apical membrane potential (ψₐ) and decreased fractional resistance (fR) in normal human and dog but had no effect on ψₐ or fR in cystic fibrosis cells. Reduction of mucosal chloride from 120 to 5 mM by replacement with gluconate increased fR of dog and normal human monolayers and depolarized ψₐ by 22 (dog) or 30 (human) mV. In cystic fibrosis monolayers, chloride replacement hyperpolarized ψₐ by 2 mV and had little effect on fR. These results suggest that the primary defect in cystic fibrosis is reduced apical membrane chloride conductance.

There is evidence that the underlying genetic defect in cystic fibrosis (CF) is a reduced permeability of epithelia to chloride (Cl). Quinton (1), the first to provide such evidence, treated sweat gland ducts with ouabain and then perfused their lumina with 50 mM NaCl or 75 mM Na₂SO₄. Large lumen-negative potentials were generated in normal tissues but only small potentials were found in CF ducts. From these data, Quinton calculated that the ratio of sodium to chloride permeability (PNa/PCl) was increased to 10 times that of normal in CF sweat ducts. Knowles et al. (2) reported similar results for in vivo nasal epithelium. Direct evidence that the increase in PNa/PCl in CF nasal mucosa was due to a reduction in Cl permeability came from Using chamber studies (3), which showed that in CF nasal mucosa the resistance was twice normal and the unidirectional CI fluxes were half those of the normal tissue. These results on sweat duct and nasal mucosa provided strong evidence that epithelial Cl permeability is reduced in CF. However, they failed to determine whether this defect lies in the trans- or the paracellular pathway.

It is now well established that epithelial cells will grow in culture to establish confluent monolayers with tight junctions separating discrete apical and basolateral cell membranes (4–8). Furthermore, the electrical properties of cultured cell sheets resemble those of the original tissue (9–12). Cells of dog tracheal epithelia when grown in culture also retain differentiated transport function (13). These monolayers resemble the original tissue in their resistance (∼400 Ω cm²), the presence of both active chloride secretion and active sodium absorption, and the stimulation of Cl secretion by a wide variety of endogenous mediators.

In this paper we have used cell culture techniques in an attempt to localize the chloride permeability defect implicated in CF. We compare ion transport by monolayers cultured from cells of dog, normal human, and CF tracheal mucosa. In particular, the properties of the transcellular pathway have been investigated using intracellular microelectrodes.

METHODS

Cell cultures were obtained as described in detail elsewhere (8, 13). In brief, strips of tracheal mucosa were digested with collagenase and the dispersed cells were plated at 2.5 or 5 × 10⁵ cells per cm² (dog) or 10⁶ cells per cm² (human) onto Nuclepore filters coated with human placental collagen. Confluent monolayers were studied in conventional Using chambers (8, 13) or with intracellular microelectrodes.

In the Using chamber studies, the transepithelial potential difference (Δψₑ) was clamped at zero and the short-circuit current (Isc) was monitored continuously on a pen recorder. Δψₑ was referenced to the mucosal solution. Transepithelial resistance (Rₑ) was calculated from the change in current in response to square-wave voltage pulses of 0.2–1 mV (duration, 5 sec; period, 20 sec). For the microelectrode studies, monolayers were kept at their spontaneous Δψₑ. Rₑ was calculated from the change in ψₑ produced by bipolar current pulses (duration, 1 sec; period, 5–10 sec; amplitude, ±40 to ±160 μA cm⁻²). The electrical potential difference across the apical membrane (Δψₐ) was measured with intracellular microelectrodes as described (14). The fractional resistance of the apical membrane (fRₐ) was determined from fRₐ = Δψₐ/Δψₑ = Rₑ/(Rₑ + Rₒ), where Δψₐ and Δψₑ refer to the changes in ψₑ and ψₐ produced by the constant current pulses and Rₑ and Rₒ refer to apical and basolateral membrane resistance, respectively.

Abbreviations: CF, cystic fibrosis; Isc, short-circuit current; P, permeability; Rₑ, Rₐ, and Rₒ, apical membrane, basolateral membrane, and transepithelial resistances; ψₑ, ψₐ, and Δψₑ, the corresponding potential differences; fR, fRₐ, fRₑ, and fRₐ the corresponding potential differences; Rₑ, fractional resistance (= Rₑ/(Rₑ + Rₒ)); PGE₂, prostaglandin E₂.
RESULTS

Cell Yield and Cytology. Details of cell yield and viability for dog and normal human cells have been described (8, 13).

A CF trachea was obtained 4 hr after the death of a 12-year-old girl. A piece of the posterior membranous portion was mounted in an Ussing chamber and found to have zero potential difference and resistance. The remainder of the trachea was used for cell isolation. Cells were collected at 30-min intervals for 2 hr. In the first 30 min, \(6 \times 10^6\) viable cells were obtained. These were mainly ciliated cells as were the \(7 \times 10^6\) cells obtained in the second 30-min period. In the two remaining 30-min periods, \(41\) and \(5 \times 10^6\) cells were obtained, respectively. Most of these were small (=7-\(\mu\)m diameter) and lacked cilia. We assume that they were basal cells. The cells were pooled and preplated for 1 hr to remove fibroblasts (13). Following preplating, \(41 \times 10^6\) cells (92%) excluded trypan blue. Four confluent monolayers were produced from these cells.

The monolayers appeared confluent 1 day after plating. Electron microscopy of 5-day-old cells revealed that they were flattened and were joined by tight junctions that separated two distinct membranes. The membrane facing the medium contained microvilli and a pronounced glyocalyx. The membrane apposed to the filter was relatively undifferentiated. The morphology of the CF cells (Fig. 1) was similar to that of normal human cells. The direction of drug action (see below) led us to identify the membrane facing the medium as the apical membrane and the membrane apposed to the filter as the basolateral membrane.

Transepithelial Electrical Properties. It took approximately 1 min to mount the tissues in the Ussing chambers. Immediately after mounting, we obtained the following values for the electrical parameters: \(I_{sc} = 2.5 \pm 0.4\ \mu\text{A/cm}^2\), \(R_1 = 486 \pm 85\ \Omega\cdot\text{cm}^2\), \(\phi_i = 1.3 \pm 0.4\ \text{mV}\) (all values are mean \pm SEM, \(n = 3\)). After 15 min, these parameters had decreased to the following steady-state values: \(I_{sc} = 1.8 \pm 0.4\ \mu\text{A/cm}^2\), \(R_1 = 380 \pm 50\ \Omega\cdot\text{cm}^2\), \(\phi_i = 0.7 \pm 0.2\ \text{mV}\). The corresponding values for "normal" monolayers are \(I_{sc} = 3.1 \pm 0.4\ \mu\text{A/cm}^2\) and \(R_1 = 436 \pm 45\ \Omega\cdot\text{cm}^2\) (mean \pm SEM of 15 filters from four humans). The \(I_{sc}\) of the CF monolayers is significantly less than that of the normal monolayers \((P < 0.05, \text{Student's } t\ \text{test})\). The resistances of the two groups are not statistically different.

The responses of CF cells to isoproterenol (10 \(\mu\text{M},\) serosal bath) together with a pair of typical responses from normal monolayers are shown in Fig. 2. With the CF filters, the responses to isoproterenol were small or nonexistent, the mean maximal increase in \(I_{sc}\) induced by isoproterenol being 0.22 \pm 0.12 \(\mu\text{A/cm}^2\). Resistance was not affected. In comparison, the maximal increase in \(I_{sc}\) in normal human tissues is 2.84 \pm 0.50 \(\mu\text{A/cm}^2\) (mean \pm SEM; five filters from four humans). Prostaglandin \(E_2\) (PGE\(_2\)) (10 \(\mu\text{M},\) serosal and mucosal baths) was added to the tissue that failed to respond to isoproterenol (Fig. 2) and it caused a transient increase in \(I_{sc}\) of approximately 2-min duration with maximal amplitude of 0.3 \(\mu\text{A/cm}^2\). Again this is approximately 1/10th of the change produced by PGE\(_2\) in normal tissues of 3.3 \pm 1.6 \(\mu\text{A/cm}^2\) (mean \pm SEM, three filters from three humans).

The conductance of the CF monolayers of 2.63 mS/cm\(^{-2}\) predicts that in the absence of a transepithelial potential

FIG. 1. Cross sections of normal (Upper) and CF (Lower) cells. (Bar = 5 \(\mu\text{m}.\))
difference, the sum of the passive ion fluxes in either direction across the cells will be $2.62 \mu \text{eq cm}^{-2} \text{hr}^{-1}$ (15; 16). The $I_{sc}$ of 1.8 $\mu \text{A cm}^{-2}$ predicts a net active transport of ions of 0.07 $\mu \text{eq cm}^{-2} \text{hr}^{-1}$. The passive ion fluxes are thus some 40 times greater than the active. Given this discrepancy, we felt it unreasonable to try to measure active ion transport using radioactive tracers; instead, we used specific transport blockers to provide indirect information about the transport processes present. All blockers were used at 0.1 mM. Mucosal amiloride and serosal ouabain inhibited $I_{sc}$ in both CF and normal tissues (Fig. 3). Serosal amiloride or mucosal ouabain were without effect on $I_{sc}$. The major difference

between CF and normal tissues is in their responses to loop diuretics. Furosemide, bumetanide, or MK-196 all inhibit $I_{sc}$ in normal tissues, and bumetanide is only effective from the serosal side of the tissue. By contrast, in CF monolayers, bumetanide failed to affect $I_{sc}$ from either the mucosal or the serosal side of the monolayer. Typical records for the effects of transport inhibitors are illustrated in Fig. 3.

**Intracellular Microelectrode Studies.** The responses in $I_{sc}$ of normal human material to mediators and transport blockers resemble the responses of dog monolayers (8, 13). Indirect evidence suggests that these responses in $I_{sc}$ are due to increased chloride secretion. To obtain more direct evidence that the cultured cells can secrete Cl, we examined the effects of isoproterenol on the cellular electrical properties.

Average values of electrical properties were obtained from multiple impalements of electrodes in dog, normal human, and CF monolayers (Table 1). In dog and normal human cells, after isoproterenol $\psi_f$ increased, $\psi_o$ depolarized, and $f_R$ decreased. This pattern of response is the same as that of intact dog tracheal epithelium (14) and suggests that isoproterenol stimulates chloride secretion by increasing apical membrane Cl permeability. In a previous study we showed that monolayers from dog retain the cell membrane Cl secretory properties of the original epithelium even though the transepithelial response to secretagogues is reduced (17). In contrast, values of $\psi_f$, $\psi_o$, and $f_R$ were unaltered by isoproterenol in the CF monolayer.

To localize the defect in chloride secretion in the CF epithelial monolayers, we examined the responses to replacing Cl in the mucosal bath by gluconate. Tissues were stimulated with isoproterenol and the mucosal Cl concentration was then reduced to 5 mM (Fig. 4). In dog, normal human, and CF monolayers, a decrease in mucosal Cl concentration produced similar increases in $\psi_f$ and $R_t$ (Table 2). In dog and normal human cells, $\psi_f$ depolarized and $f_R$ increased. These are the responses expected from a Cl-permeable membrane and are the same as the changes seen in the original dog tracheal epithelium (14). In contrast, $\psi_f$ of the CF monolayer hyperpolarized slightly and $f_R$ did not change significantly.

**DISCUSSION**

Monolayers derived from tracheal mucosa of dogs or normal humans show similar electrical properties. Monolayers de-

---

**Table 1. Effect of isoproterenol (5 $\mu$M, serosal side) on cellular electrical properties**

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>$\psi_f$, mV</th>
<th>$\psi_o$, mV</th>
<th>$f_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>6</td>
<td>0.4 ± 0.3</td>
<td>-38 ± 3</td>
<td>0.71 ± 0.06</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>6</td>
<td>1.2 ± 0.4*</td>
<td>-23 ± 3*</td>
<td>0.24 ± 0.05*</td>
</tr>
<tr>
<td>Normal human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16</td>
<td>0.1 ± 0.2</td>
<td>-35 ± 2</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10</td>
<td>0.7 ± 0.2*</td>
<td>-23 ± 1*</td>
<td>0.34 ± 0.02*</td>
</tr>
<tr>
<td>CF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>4</td>
<td>0.2 ± 0.1</td>
<td>-30 ± 1.0</td>
<td>0.79 ± 0.05†</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10</td>
<td>0.3 ± 0.1</td>
<td>-32 ± 2.5†</td>
<td>0.76 ± 0.03†</td>
</tr>
</tbody>
</table>

Values were obtained from six cell sheets cultured from dogs, four from normal humans, and one from a patient with CF. $n$, Number of electrode impalpements under each condition. For the dog the values before and after addition of drug were obtained from a single impalement in each cell sheet. For the normal human and the CF patient the values represent multiple impalpements obtained in each monolayer.

* $P < 0.05$ compared with baseline by paired $t$ test for the dog and unpaired $t$ test for the humans.

† $P < 0.005$ compared with the value under same condition in the normal human by unpaired $t$ test.
The behavior of CF absence of derived from both bumetanide and amiloride. Bumetanide acts only from the serosal side, amiloride only from the mucosal side. This suggests that both chloride secretion and sodium absorption are present. In humans, about 40% of the baseline Isc persists in the presence of a combination of bumetanide and amiloride. As discussed elsewhere (8), in dog tracheal epithelium amiloride blocks only ~50% of active Na absorption and bumetanide does not remove all active Cl secretion. The residual I sc in cultured cells is therefore probably due to a combination of amiloride-insensitive Na absorption and bumetanide-insensitive Cl secretion.

Several types of endogenous mediators stimulate I sc across dog and normal human cells in culture (8, 13). This stimulation is probably due to increased chloride secretion. Thus, sodium absorption across intact airway epithelia is insensitive to mediators (8). Also, in cultured dog cells the mediator-induced increases in I sc are greatly reduced in Cl-free (glucuronate) medium (unpublished results). Finally, our intracellular recordings of the response to isoproterenol show that this drug decreases fR and depolarizes $\psi_s$ to $-23$ mV, a value close to the expected equilibrium potential for Cl (Table 1). Thus, in dog and normal human cells, the response to isoproterenol seems to be an increase in the apical membrane Cl permeability leading to an increase in net transepithelial Cl secretion. In contrast to normal human cells, the I sc of CF cells is unaffected by bumetanide and the responses to isoproterenol and PGE 2 are reduced to about 1/10th of normal. Furthermore, isoproterenol does not change fR or $\psi_s$.

Table 2. Effect of mucosal chloride substitution on cellular electrical properties

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$\psi_o$, mV</th>
<th>$\psi_s$, mV</th>
<th>$R_o$, $\Omega$cm$^2$</th>
<th>$f_R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>6</td>
<td>0.6 ± 0.4</td>
<td>$-27 \pm 3$</td>
<td>47 ± 7 0.22 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>124 mM Cl</td>
<td>5</td>
<td>7.0 ± 0.8*</td>
<td>$-5 \pm 4^*</td>
<td>74 ± 11 0.49 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>5 mM Cl</td>
<td>11</td>
<td>0.9 ± 0.2</td>
<td>$-23 \pm 1$</td>
<td>284 ± 37 0.36 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Normal human</td>
<td>3</td>
<td>7.8 ± 0.3*</td>
<td>$+7 \pm 2^*</td>
<td>357 ± 37 0.57 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>3</td>
<td>0.4 ± 0.2</td>
<td>$-28 \pm 1$</td>
<td>111 ± 7 0.69 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>124 mM Cl</td>
<td>5</td>
<td>9.1 ± 1.2*</td>
<td>$-30 \pm 2^*</td>
<td>140 ± 5 0.80 ± 0.03*</td>
<td></td>
</tr>
</tbody>
</table>

Values were obtained from six cell sheets from dogs, three from normal humans, and one from a patient with CF. n, Number of electrode impalements for each group. Values obtained with normal (124 mM) and decreased (5 mM, gluconate substitution) Cl concentrations were obtained during single cellular impalements.

*P < 0.005 compared with value in 124 mM Cl by paired t test.
†P < 0.005 compared with value from the normal human with 5 mM Cl by unpaired t test.

These results suggest that in CF cells Cl secretion and its stimulation by secretagogues are absent or greatly reduced.

To localize the defect responsible for impaired chloride secretion in CF cells we used intracellular microelectrodes to study the effects of reducing mucosal Cl concentration. When most of the mucosal Cl was replaced by gluconate, dog and normal human monolayers showed the response expected for Cl-selective apical membranes: $\psi_s$ depolarized and fR increased. In contrast, the same treatment in CF monolayers caused $\psi_s$ to hyperpolarize slightly without much change in fR. These results are consistent with a reduced Cl permeability of the apical membrane in CF.

The mechanisms behind the hyperpolarization of $\psi_s$ seen in CF cells on replacing chloride are uncertain. As Cl conductance seems to be absent, amiloride-sensitive sodium channels should result in the apical membrane of CF cells being highly Na selective. We speculate that the activity coefficient for sodium gluconate is less than that for NaCl. In this case replacing NaCl by an equivalent amount of sodium gluconate should lead to a reduction in the Na activity gradient across a Na-selective membrane. This could account for the hyperpolarization of $\psi_s$ and small increase in fR seen in CF cells.

Indirect evidence suggests that the ion selectivity of the transcellular pathway is unaffected in CF. In unstimulated dog tracheal epithelium, the conductance of the transcellular pathway accounts for ~85% of the total tissue conductance (18). In cultured cells, $R_t$ is much the same as in the intact epithelium but the I sc is only about 1/10th that of the original tissue. This suggests that the conductance of the transcellular pathway is reduced in culture and that the paracellular conductance may be much greater than the apical membrane conductance ($G_a$). Thus, changes in $\psi_s$ in response to changes in the ion content of mucosal solution may reflect mainly the ion selectivity of the transcellular route. When chloride was reduced to 5 mM by replacement with gluconate, the increase in $\psi_s$ were the same for dog, normal human, and CF cells (Table 2). Thus, the ion selectivity of the paracellular pathway may be little altered in CF. In sweat ducts and nasal mucosa (1, 2), the large lumen-negative potential differences induced by mucosal Cl replacement may reflect a relatively greater contribution of $G_a$ to $G_t$ in normal tissue.

Table 3. Effect of mucosal Cl substitution on tissue conductance

<table>
<thead>
<tr>
<th></th>
<th>$G_t$, nS</th>
<th>$G_a$, nS</th>
<th>$G_b$, nS</th>
<th>$G_e$, nS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>6</td>
<td>1.2</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>124 mM Cl</td>
<td>5</td>
<td>1.3</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>5 mM Cl</td>
<td>11</td>
<td>1.4</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Normal human</td>
<td>3</td>
<td>1.5</td>
<td>1.0</td>
<td>0.7</td>
</tr>
<tr>
<td>CF</td>
<td>3</td>
<td>1.6</td>
<td>1.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Values were obtained from six cell sheets from dogs, three from normal humans, and one from a patient with CF. n, Number of electrode impalements for each group. Values obtained with normal (124 mM) and decreased (5 mM, gluconate substitution) Cl concentrations were obtained during single cellular impalements.

*P < 0.005 compared with value in 124 mM Cl by paired t test.
†P < 0.005 compared with value from the normal human with 5 mM Cl by unpaired t test.

Unlike chloride secretion, there seems to be little change in sodium absorption in CF. This is illustrated in Fig. 3, where the decrease in $I_{sc}$ induced by amiloride is similar in both CF and normal material. Our small sample size does not permit a more detailed comparison of Na transport rates.

There seem to be three possible chloride channel defects that singly or in combination could explain the reduced apical membrane Cl permeability in CF. Chloride channels may be present in reduced numbers, they may have structural alterations leading to reduced ability to transport Cl, or their
regulation may be faulty. A defect in regulation is suggested by the results of Sato and Sato (19). They found that methacholine induced the same flow rates from normal and CF sweat glands and that isoproterenol caused the same elevation of cyclic AMP in control and CF glands and stimulated sweat secretion in normal glands but did not induce flow from CF glands. Our results with isoproterenol could also reflect a defect in cyclic AMP-dependent regulatory processes in CF. In addition, we found that the response to PGE$_2$ was reduced in CF. PGE$_2$ resembles isoproterenol in that it seems to stimulate Cl secretion across dog tracheal epithelium by raising intracellular cyclic AMP levels (20).

In conclusion, the work described here indicates that the basic defect in CF is a reduction in the permeability of epithelial apical membranes to chloride. Our data support the growing body of evidence that epithelial cells retain differentiated transport function in culture and we have shown that, although the postmortem CF tissue lacked measurable resistance and potential difference, cultured cell sheets exhibited active ion transport and high trans-epithelial resistance. We suggest that cell culture will continue to be a valuable approach to the study of the Cl channel defect in cystic fibrosis.

We thank Dr. J. A. Nadel for his support and interest and Drs. Brian Davis and Christopher J. Newth for helping us obtain the CF tissue. We are also grateful to Ella Highland, Timothy Ruppert, and Phil Karp for technical assistance. This study was supported in part by the National Institutes of Health Program Project Grant HL-24136 and grants from the National Cystic Fibrosis Foundation, Cystic Fibrosis Research, Inc., and National Medical Enterprises, Inc. M.J.W. is an Established Investigator of the American Heart Association.