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Altered chloride metabolism in cultured cystic fibrosis skin fibroblasts

(chloride efflux/transport)

PAMELA M. MATTES*, PETER C. MALONEY, AND JOHN W. LITTLEFIELD

Department of Physiology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

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ABSTRACT **An abnormal regulation of chloride permeability has been described for epithelial cells from patients with cystic fibrosis (CF). To learn more about the biochemical basis of this inherited disease, we have studied chloride metabolism in cultured CF fibroblasts by comparing the efflux of $^{36}Cl^-$ from matched pairs of CF and normal fibroblasts. The rate constants describing $^{36}Cl^-$ efflux did not differ between the two cell types, but in each of the four pairs tested the amount of $^{36}Cl^-$ contained within CF cells was consistently reduced, by 25–30%, relative to normal cells. Comparisons of cell water content and $^{22}Na^+$ efflux showed no differences between the two cell types, suggesting that overall intracellular chloride concentration is lower than normal in CF fibroblasts. Such data suggest that the CF gene defect is expressed in skin fibroblasts and that this defect may alter the regulation of intracellular Cl^- concentration, perhaps through changes in Cl^- permeability.**

Cystic fibrosis (CF) is a lethal, autosomal recessive disease that affects approximately 1 in 2000 live births in the United States. The clinical manifestations of CF are well described, many relating to fluid and electrolyte imbalances in exocrine tissues, yet the underlying biochemical defect is not understood. Current evidence suggests that certain CF exocrine gland epithelia have a reduced permeability to the chloride ion. This was proposed first by Quinton (1), whose studies of the isolated and perfused CF sweat gland reabsorptive duct demonstrated that the abnormally high transepithelial voltage in this tissue was due to a reduced electrodiffusive movement of Cl^-. Knowles *et al.* (2, 3) have arrived at a similar conclusion with regard to Cl^- movement across nasal epithelium. A second line of investigation now centers on cultured epithelial cells (4, 5), and these studies have indicated that reduced Cl^- permeability in the affected epithelia may reflect defects at a cellular level in the regulation of Cl^- channel activity, without structural changes in the channel itself.

Given the evidence that CF leads to altered metabolism of Cl^- in affected tissues and cells, it seemed reasonable to extend the analysis to cell types more amenable to direct study. For this reason, we have examined cultured skin fibroblasts, an adaptable, easily controlled, and relatively long-lived experimental system that has proven of value to studies of inborn errors of metabolism, even when the fibroblast itself is not the subject of disease pathology. If Cl^- transport is altered in CF epithelia, parallel identification of the defect in CF fibroblast lines could be useful in advancing the study of CF at the cellular and molecular levels.

We examined $^{36}Cl^-$ efflux under steady-state conditions from four pairs of CF and normal (N) fibroblasts that had been matched for donor and culture age. In each pair the CF cell line showed a depression in the curve for isotope efflux when compared to its control partner. Further study has now attributed these findings to a decreased overall concentration of Cl^- in the CF cell. Since the preliminary report of this work (6), studies in another laboratory (7) have also implicated an abnormal electrolyte metabolism in CF fibroblasts, although in that instance the experimental findings differed from those outlined here.

MATERIALS AND METHODS

Cell Culture. CF fibroblast lines were obtained using forearm punch biopsies taken from donors who were patients at the Johns Hopkins Cystic Fibrosis Center; patient donors were unrelated to one another. The age-matched control donors had no known family history of the disease. Explants were grown in 60-mm culture dishes (Falcon) using minimal essential medium with nonessential amino acids (GIBCO), 15% fetal calf serum (KC Biological, Lenexa, KS), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). After ≈5 weeks, CF and N lines had reached a confluent density near 5×10^6 cells per 60-mm dish. At that time aliquots of each cell line either were stored frozen in liquid N_2 for future use or were subcultured by seeding at 4×10^5 cells per 75-cm^2 flask (Corning) in medium with 10% fetal calf serum. Growing cells were fed every 3 days and passed every 10–14 days. Cultures were found to be mycoplasma-free in assays performed by Flow Laboratories.

Previous work (ref. 8; P.M.M., unpublished observations) has suggested that during extended growth *in vitro* CF fibroblasts encounter senescence earlier than normal. Consequently, the present work has taken care to compare CF and N cell lines similar in several respects. Cells were derived from individuals of the same age (±2 years). Also, each pair has been propagated in parallel, beginning with the explant, so that each comparison has used cells of comparable generation number. Finally, all experiments were done with cells younger than 25 population doublings.

Ion Transport Measurements. For ion transport studies fibroblasts were seeded in 35-mm Petri dishes (Falcon) 6–9 days before an experiment and were fed every 2–3 days thereafter. Assays were performed using confluent cultures. CF and N cells gave the same protein yield per dish (in 31 experiments, the ratio CF/N ± SEM was 0.94 ± 0.02).

Chloride efflux was estimated under steady-state conditions as described by Pato *et al.* (9) using an assay temperature of 25–27°C. This temperature was chosen in an effort to reduce the relative contribution of carrier-mediated movements to the total Cl^- flux. In routine work, the cells attached to a 35-mm dish were washed with physiological salt solution (50 mM Hepes, sodium salt/130 mM NaCl/5.4 mM KCl/1.8 mM $CaCl_2$/5.5 mM glucose/10 mM Na_2HPO_4/0.8 mM

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Abbreviations: CF, cystic fibrosis; N, normal; 3-*O*-MeGlc, 3-*O*-methyl-D-glucose.

*Present address: Department of Pediatrics, University of Connecticut Health Center, Farmington, CT 06032.

$MgSO_4$, pH 7.4) and then loaded with tracer by a 2-hr incubation with gentle swirling in 1 ml of physiological salt solution with added $^{36}Cl^-$ [5–10 μCi/ml, 36–72 μCi/mmol (1 Ci = 37 GBq); New England Nuclear]; further incubation (to 3 hr) did not change the level of isotope taken up by cells. After loading, the medium was removed by aspiration, the cells were washed rapidly by three successive immersions in isotonic sucrose, and the assay of efflux was started by exposing the cells to 1 ml of the salt solution without added tracer. Over the next 20–30 min, the "washout" of $^{36}Cl^-$ was measured by removing the solution at intervals, each time replacing it with another 1 ml of fresh fluid. At the end of the experiment, the cells were dissolved in 0.2 M NaOH to measure protein and residual radioactivity. Results were expressed (per unit protein) as the isotope remaining in cells at each time. An experiment always compared (in duplicate) the behavior of age-matched CF and N fibroblasts; duplicate time courses usually agreed within 10% or less.

Double-label experiments to monitor $^{22}Na^+$ and $^{36}Cl^-$ efflux were done in the same way, except that the cells were exposed to $^{22}Na^+$ (10–15 μCi/ml, 61–91 μCi/mmol; New England Nuclear or Amersham) and $^{36}Cl^-$ (10 μCi/ml, 72 μCi/mmol) during the loading period. $^{22}Na^+$ was determined in a γ counter, and $^{36}Cl^-$ was measured by liquid scintillation spectroscopy, with corrections for the contribution by $^{22}Na^+$.

Kinetic Analysis. Results were consistent with the efflux of $^{36}Cl^-$ from a simple two-compartment system (9) represented by the equation:

$$Y = A_{app} \cdot e^{-k_a t} + B_{app} \cdot e^{-k_b t}.$$

Y gives isotope content at time t, A_{app} and B_{app} represent the apparent zero-time isotope capacities for compartments A and B, and k_a and k_b are rate constants governing isotope efflux from A and B, respectively. Kinetic parameters for each compartment were resolved graphically by "curve peeling," a method (10) in which B is evaluated by the zero-time Y intercept of a line extrapolated using late values (here, $t > 3$ min) from the semilogarithmic plot of isotope content versus time. Having calculated k_b from the negative slope of the extrapolated line, the contribution of compartment B ($B_{app} \cdot e^{-k_b t}$) at each time was subtracted from Y, and the remainder ($A_{app} \cdot e^{-k_a t}$) was used to generate values of A and k_a. Such kinetic analysis cannot identify the biological equivalents of compartments A and B without further work, nor can one assume that compartments identified by studies of $^{36}Cl^-$ efflux correspond to the compartments (A' and B') found by analysis of $^{22}Na^+$ efflux. Nevertheless, it is customary to assume that the more rapidly emptying compartment represents cytosol and that more slowly turning-over compartments reflect one or more subcellular organelle(s) or perhaps bound material (7, 9, 10). In this regard, it should be noted that the values derived in this analysis describe isotope efflux from parallel and independent compartments, and for simplicity we have tabulated the data in this way. But if efflux occurs in series (from B to A and then to the medium), a backflux from A to B occurs, and the relative compartment sizes must be modified accordingly (10). This correction (10) did not significantly alter our experimental findings (see below).

Cell Water Measurements. Cell volume was measured as the volume of distribution of the D-glucose analog 3-*O*-methyl-D-glucose (3-*O*-MeGlc). Facilitated and passive diffusion of this nonphosphorylated analog establishes an internal 3-*O*-MeGlc concentration equal to the external concentration (11). Fibroblasts were seeded in 35-mm dishes ≈1 week before assay, and confluent monolayers were washed with a glucose-free physiological salt solution (pH 7.4) before incubation at room temperature with the same solution containing 3-*O*-[^{14}C]MeGlc (5 μCi/ml, 500 μCi/mmol) at 10 mM. A preliminary experiment under these conditions showed that 3-*O*-MeGlc was readily taken up at 27°C (an initial rate of 2.4 nmol/min per mg of cell protein) and that no further uptake occurred after about 30 min ($t_{1/2}$ for equilibration was 7 min). In routine work, samples were taken at steady state (40–60 min), the medium was removed, and cells were washed with iced phosphate-buffered saline (pH 7.4) that contained 1 mM phloridzen (12) to prevent loss of internal 3-*O*-MeGlc by facilitated diffusion. On solubilization with 0.2 M NaOH, aliquots were taken for determination of radioactivity and protein.

RESULTS

Chloride Transport by CF and N Fibroblasts. Following the reports of chloride impermeability in CF exocrine tissues, we examined the transport of this anion using CF fibroblast cultures. The method chosen involved measurement of $^{36}Cl^-$ efflux from isotope-loaded cells under conditions in which the labeled anion was diluted into an effectively infinite extracellular pool. This allows estimates of the total cellular capacity for Cl^- and the rate constants that describe Cl^- efflux from the cell.

The data in Fig. 1 illustrate the basic finding in such studies. Each panel shows a semilogarithmic plot of isotope content versus time for a different matched pair of CF and control fibroblast lines. In each case the curve describing $^{36}Cl^-$ efflux from the CF cells falls below the curve for efflux from N cells. To understand the kinetic origin of this disparity, we analyzed the data by curve peeling, as noted in *Materials and*

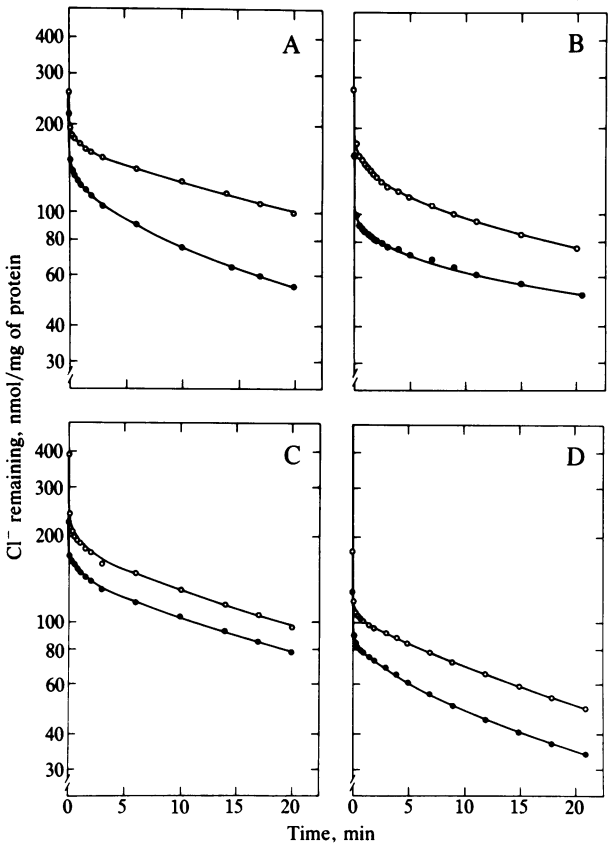

FIG. 1. Time course of $^{36}Cl^-$ efflux from age-matched pairs of CF and N fibroblasts. In four separate experiments, pairs of CF and N fibroblasts were incubated in physiological salt solution with added $^{36}Cl^-$. After isotopic equilibrium was reached, cells were reincubated in the same salt solution but without added tracer, and samples were obtained at the indicated times to monitor $^{36}Cl^-$ efflux. ○, N fibroblasts; ●, CF fibroblasts.

Methods. Data from that analysis are shown in Table 1, which summarizes experiments that compared $^{36}Cl^-$ efflux from each of the four pairs of CF and N cell lines. The data are expressed as relative ratios (CF/N) because of day-to-day variation in absolute values (see below). Judging by the examples given in other studies using human fibroblasts (7, 9), a significant fraction of cell Cl^- (55–70%) can be found in the more slowly emptying compartment (*B*). Our results were in general agreement with such observations, and about 80% of the total Cl^- was contained in this slowly emptying compartment, for either CF or N cells (Table 1 legend) [this fraction fell to 75% if the calculations assumed efflux from compartments in series (see *Materials and Methods*)]. Although the internal distribution of Cl^- was the same for the two cell types, the CF fibroblasts consistently displayed a lower total Cl^- content when compared to control cells, and for compartments *A* and *B* the CF lines had Cl^- levels 71–76% of their paired controls (75% ± 4% and 75% ± 2% for compartments *A* and *B*, respectively, assuming efflux in series). Because there were no comparable differences in the values of either k_a or k_b, we have concluded that the disparity between the $^{36}Cl^-$ efflux curves of CF and control cells reflected a lower Cl^- capacity of CF fibroblasts.

Cell Volume. In the next experiments we measured the volume of CF and N cells to determine the extent to which the changes in $^{36}Cl^-$ content were correlated with changes in average Cl^- concentration. Cell volume was taken as the volume of distribution of 3-*O*-MeGlc (11), since this method allowed us to study monolayer cultures without disrupting cell morphology. To validate the technique for the conditions used here, we first performed a control experiment in which steady-state 3-*O*-MeGlc content was determined for a N line (from pair A, Fig. 1) using substrate concentrations (0.5–10 mM) that spanned the Michaelis constant (K_m) for transport (13). This experiment showed that there was a direct and linear relationship between internal 3-*O*-MeGlc content and its external concentration (data not shown). The slope of the line relating these quantities was 5.3 μl/mg of protein, which gave an estimate of cell volume close to the value (4.4 μl/mg of protein) found by others who have studied human fibroblasts (14).

Table 2 summarizes data obtained in 12 other experiments that measured 3-*O*-MeGlc distributions for the four pairs of CF and control lines. For each cell line, 3-*O*-MeGlc taken up at steady state varied nearly 2-fold from one experiment to the next, indicating that valid comparisons required the use of matched pairs of lines tested at the same time. But despite this day-to-day variation, in any single experiment CF and N fibroblasts had comparable 3-*O*-MeGlc contents, corresponding to mean values of 4.96 and 5.20 μl/mg of protein. Therefore, because cell volume does not differ between CF and control fibroblasts, the lowered $^{36}Cl^-$ capacity of CF cells corresponded to a lowered overall Cl^- concentration.

Table 1. Kinetic parameters describing $^{36}Cl^-$ efflux from CF and N fibroblasts

Fibroblast pair	Compartment size (CF/N)		Rate constant (CF/N)	
	A_{app}	B_{app}	k_a	k_b
A (8)	0.67 ± 0.08	0.77 ± 0.03	1.10 ± 0.08	1.21 ± 0.07
B (3)	0.59 ± 0.10	0.74 ± 0.06	0.88 ± 0.06	1.03 ± 0.08
C (4)	0.73 ± 0.05	0.77 ± 0.01	1.09 ± 0.13	1.02 ± 0.04
D (4)	0.85 ± 0.14	0.75 ± 0.04	1.26 ± 0.27	1.11 ± 0.04
A–D	0.71 ± 0.05	0.76 ± 0.02	1.10 ± 0.08	1.14 ± 0.04

Isotopic efflux was performed by using the fibroblast pairs (A, B, C, D) noted in Fig. 1; for each pair of cell cultures the number of experiments is given in parentheses. Data were analyzed assuming two parallel cellular compartments from which efflux occurred by first-order kinetics (7, 9). In each experiment compartment sizes and associated rate constants were evaluated by curve peeling. Data are given as mean ratios ± SEM (CF/N) of those values. For N cells mean absolute values were A_{app}, 32.2 nmol/mg of protein; B_{app}, 138 nmol/mg of protein; k_a, 0.615 per min; k_b, 0.034 per min. For CF cells mean absolute values were A_{app}, 23.4 nmol/mg of protein; B_{app}, 108 nmol/mg of protein; k_a, 0.635 per min; k_b, 0.039 per min.

Table 2. Cell volume measurements for CF and N fibroblasts

Fibroblast pair	μl/mg of protein		CF/N
	CF	N	
A	3.65 ± 0.19	3.97 ± 0.08	0.92
	5.95 ± 0.20	6.15 ± 0.33	0.97
	6.85 ± 0.33	7.33 ± 0.62	0.93
B	5.10 ± 0.33	4.57 ± 0.27	1.12
	4.20	4.58	0.92
	5.04 ± 0.09	4.82 ± 0.22	1.04
C	4.79	5.43	0.88
	4.67 ± 0.06	4.86 ± 0.59	0.96
	3.92 ± 0.34	4.65 ± 0.61	0.84
D	4.46 ± 0.08	4.77 ± 0.06	0.94
	5.34 ± 0.07	5.84 ± 0.22	0.91
	5.57 ± 0.17	5.44 ± 0.11	1.02
A–D	4.96 ± 0.26	5.20 ± 0.26	0.96 ± 0.02

Cell volume was measured in 12 experiments. The indicated pairs of CF and N fibroblasts were incubated with 10 mM 3-*O*-MeGlc to determine cell volume. Each row gives results from a separate experiment. In each experiment the values represent the means ± SEM of three to six points; values without SEM are the means of duplicate samples.

Efflux of Sodium. The finding that CF fibroblasts had a decreased capacity for Cl^- (Table 1) but normal cell volume (Table 2) implied that the net internal Cl^- concentration of CF cells was reduced. To examine the possibility that the levels of other ions were also lowered in CF cells, we studied $^{22}Na^+$ efflux. This choice was made because $^{22}Na^+$ and $^{36}Cl^-$ are conveniently measured together in double-label experiments and also because Na^+ and Cl^- are each at low levels relative to K^+ or to organic anions. Using two pairs of CF and N fibroblasts (pairs A and C, Fig. 1; Tables 1 and 2), we performed experiments in which the efflux of both isotopes was monitored at the same time. As illustrated by Fig. 2, these experiments confirmed that $^{36}Cl^-$ efflux from CF cells differed from that of control cells. However, the curves describing $^{22}Na^+$ efflux did not differ one from the other. During this work we often found considerable scatter, even in a single experiment, for the initial phases of $^{22}Na^+$ efflux, so that reliable information was not available from the early parts of these curves (not shown). For this reason we relied only on data derived from later times, when contributions from the "fast" (rapid turnover) component(s) were negligible. These larger compartments (for $^{36}Cl^-$ and $^{22}Na^+$) were estimated as usual, and in 14 experiments the $^{36}Cl^-$ space was calculated relative to that of $^{22}Na^+$ for the same cell. For N cells this ratio ($^{36}Cl^-/^{22}Na^+$) was 5.20 ± 0.20, whereas in the paired CF lines the ratio was lowered to 3.93 ± 0.26 (means ± SEM; $P < 0.005$, unpaired *t* test). When the isotope data were treated separately, we found no differences with regard to the $^{22}Na^+$ space (the ratio CF/N was 1.1 ± 0.09), in contrast to the data for $^{36}Cl^-$ (0.79 ± 0.03 for the ratio CF/N). These findings indicate that the abnormality in overall Cl^- concentration was specific for this anion.

DISCUSSION

Electrophysiological experiments have established that epithelia in certain CF exocrine tissues show defective permeabilities to Cl^- (1–3), and in at least some instances this change has been attributed to an abnormal regulation of Cl^- channel activity (4, 5). The work presented here has extended

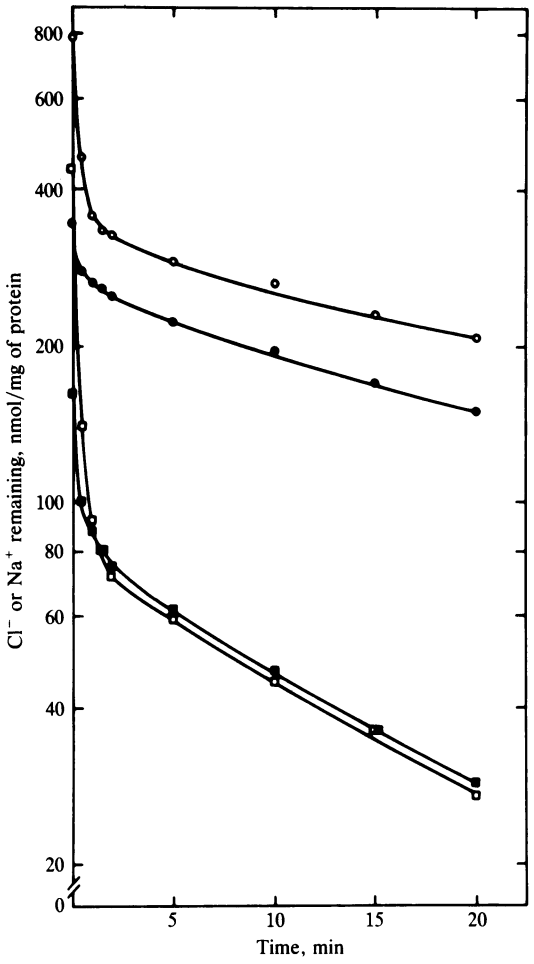

FIG. 2. $^{22}Na^+$ and $^{36}Cl^-$ efflux from age-matched CF and N fibroblasts. A pair of CF and N fibroblasts (pair A, Fig. 1) was incubated for 2.5 hr in physiological salt solution containing $^{22}Na^+$ and $^{36}Cl^-$, and the efflux of both isotopes was measured. ○, Control $^{36}Cl^-$; ●, CF $^{36}Cl^-$; □, control $^{22}Na^+$; ■, CF $^{22}Na^+$.

these observations by asking if Cl^- metabolism in CF fibroblasts is affected, using an experimental design that allowed studies of $^{36}Cl^-$ efflux under steady-state conditions (7, 9). For CF and N cells, the approach has given estimates of the total cellular capacity for Cl^- and of the unidirectional rate constant(s) needed to describe the efflux process (6, 7, 9). In agreement with earlier studies (7, 9), efflux of $^{36}Cl^-$ from human fibroblasts could be analyzed as a two-component system in which the internal compartments were distinguished from each other by kinetic and capacity terms (Table 1). In our work we did not find differences between CF and N fibroblasts with respect to the efflux rate constants k_a and k_b, but this may not be surprising. The measurements made in CF epithelia suggest that the activity or regulation of a Cl^- conductive pathway has been altered, and although fibroblasts have not been examined in this context, it is possible that in this cell the conductive pathways for Cl^- mediate only a small fraction of the total transmembrane Cl^- flux. For example, in the Ehrlich ascites tumor cells isotopic permeability to Cl^- is 20-fold higher than that estimated by specific conductance measurements (15); a similar difference is found in mammalian heart (16). These observations suggest that, at least in some cell types, most of the transmembrane Cl^- flux in the steady state occurs by a neutral exchange with other anions, such as Cl^-:HCO_3^- antiport (16), or by cotransport with cations, as in Na^+,K^+/2Cl^- symport (17). For this reason, assays of $^{36}Cl^-$ efflux may not reveal differences in Cl^- conductive pathways across fibroblast membranes. Although we did not find differences in rate constants describing efflux, others have reported (7) a 25% reduction in one of the rate constants (k_a) describing $^{36}Cl^-$ efflux from CF fibroblasts (1.0 versus 0.75 per min for k_a), with no apparent differences in cell capacities for Cl^-. We do not understand why that work contrasts with our own, but explanations may lie in the somewhat different experimental techniques and temperatures (37°C versus 27°C). Our experience also suggests that there must be careful control over variables related to cell aging and cell culture in order to have confidence in changes of this magnitude.

The presence of two internal compartments was needed to adequately describe the kinetics of $^{36}Cl^-$ efflux (Table 1), and in CF and N cells the larger compartment contained about 80% of the total Cl^-. Although our analyses did not suggest differences in the relative sizes of these compartments, when comparisons were made between CF and control cells there were consistent differences in the Cl^- capacities of smaller and larger compartments (Table 1). Cell volume, as judged by the distribution of 3-*O*-MeGlc, was the same for CF and control cells (5.1 μl/mg of protein; Table 2). Therefore, since presumably 3-*O*-MeGlc and $^{36}Cl^-$ have access to the same spaces, the different Cl^- capacities must reflect a lowered overall Cl^- concentration within CF cells. In N cells the total Cl^- capacity (170 nmol/mg of protein; Table 1 legend) would be equivalent to an average internal concentration of 33.4 mM, and the corresponding value for CF fibroblasts (132 nmol/mg of protein) would be 25.8 mM. Such estimates are in general agreement with measurements from other laboratories, which report that normal fibroblasts have a total cellular Cl^- of 200–240 nmol/mg of protein (7, 9). To our knowledge, decreased Cl^- concentration in cultured CF cells has not been reported before, although data presented by Stutts *et al.* (18) did show a 10% lower level of Cl^- in cells cultured from CF nasal epithelium. This difference was not statistically significant (18), but our findings suggest that intracellular Cl^- should be examined more closely in affected epithelial cells.

The link between reduced Cl^- levels in fibroblasts and the genetic defect in CF is not evident from our work, but an altered membrane permeability to Cl^- would be consistent with our findings. Thus, if rates of efflux are unaffected (Table 1), then reduced capacity for Cl^- should be due to changes of influx. We have not examined this question directly, but it is of interest that Stutts *et al.* (18) found that transport of $^{36}Cl^-$ into cultured CF nasal epithelial cells was reduced by about one-third. A lowered cytoplasmic Cl^- concentration has other implications as well. For example, to preserve electrical neutrality an overall reduction of cell Cl^- must be paralleled by similar decreases of cations or increases of anions. In the experiments we performed (Fig. 2 and text) the estimated Na^+ levels appeared not to change, and we presume that ionic balance is maintained either by reduced cell K^+ (such small changes would not have been detectable) or by increases in anions such as phosphate or bicarbonate. Perhaps the most significant impact of our findings is that at least one aspect of the CF defect is manifest in cultured skin fibroblasts, a cell type not known to be involved in the disease process. If the actual gene defect is of a regulatory nature, as recent studies suggest (4, 5), fibroblasts may be useful for the biochemical and molecular study of the defective protein(s).

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