A Cystic Fibrosis Phenotype in Cells Cultured from Sweat Gland Secretory Coil

ALTERED KINETICS OF $^{36}$Cl EFFLUX*

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(Received for publication, February 13, 1990)

As a step toward understanding the metabolic consequences of the cystic fibrosis (CF) mutation, we have examined the kinetics of $^{36}$Cl efflux in cells cultured from sweat glands, a tissue that is affected in the disease. Epithelial cells, derived from the secretory coil of sweat glands of CF and control individuals, were cultured in serum-free medium, and primary cultures used for efflux experiments. Cell layers were equilibrated with Na$^{36}$Cl in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered balanced salt solution for 45 min at 37 °C, washed in 0.25 M sucrose, and incubated in nonradioactive buffer for measurement of $^{36}$Cl efflux. Efflux from CF and control cells followed biphasic kinetics and was described by the equation $Y = Ae^{−αt} + Be^{−βt}$. All efflux was inhibited at 6 °C. The fast component of efflux, $Ae^{−αt}$, of both control and CF cells was inhibited by the anion channel blockers 4,4'-diisothiocyanato-2,2'-stilbene disulfonic acid, $β$-adrenergic agonists and by protein kinases A and C. The precise role of the ATP-binding site and several target sequences for phosphate transport proteins. In two-thirds of the CF alleles which had been determined; it may serve to regulate the function of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is located in a region of the protein which also contains a putative "reverse genetics" (23-25). The gene encodes a large membrane protein of 1480 amino acids, named the Cystic Fibrosis Chloride channel, rather than be the ion channel itself (24). Defective chloride conductance was first observed by Quinton (4, 5) in CF sweat gland ducts. Further electrophysiology studies have revealed a similar chloride permeability defect in cells cultured from sweat gland ducts (6) and from respiratory epithelium (7-10). Patch-clamp studies have shown that chloride channels normally activated by $β$-adrenergic agonists and by protein kinases A and C are not responsive in CF airway epithelial cells (11-16). Abnormal function of chloride channels may explain the altered water flow and electrolyte concentration in several CF tissues, which in turn contribute to CF pathology. Defects in chloride transport have also been reported in CF intestine (11, 12), lymphoblasts (19), and cultured fibroblasts (20-22).

The CF gene has recently been identified by the strategy of "reverse genetics" (23-25). The gene encodes a large membrane protein of 1480 amino acids, named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is homologous to a number of known bacterial and eukaryotic transport proteins. In two-thirds of the CF alleles which had been examined, the mutation was found to be deletion of the codon for phenylalanine at position 508. This residue is located in a region of the protein which also contains a putative ATP-binding site and several target sequences for phosphate addition by protein kinases A and C. The precise role of the cystic fibrosis gene product in chloride transport has not yet been determined; it may serve to regulate the function of the chloride channel, rather than be the ion channel itself (24).

Investigation of the metabolic defect in CF has been hindered by the lack of a suitable animal or cell culture model that displays a consistent and easily measured CF phenotype. Because exocrine glands are generally difficult to obtain and to culture, differences between normal and CF chloride transport have been sought in more readily available material. Using cultured human skin fibroblasts, differences have been found in $^{36}$Cl accumulation (91), kinetics of $^{36}$Cl efflux (90), and cAMP stimulation of chloride-conductive channels (22). The differences, though statistically significant, are not very pronounced, perhaps because fibroblasts (a cell type not

*This work was supported in part by a Research Development Program Center of the Cystic Fibrosis Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†A trainee of the UCLA Intercampus Medical Genetics Program under Grant GM 08243 during part of the period covered by these studies.

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§The abbreviations used are: CF, cystic fibrosis; DPC, diphenylamine 2-carboxylate; DIDS, 4,4'-diisothiocyanato-2,2'-stilbene disulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Na$^{36}$Cl, sodium-36 chloride; Na$^{36}$K-ATPase, sodium-potassium ATPase; Na$^{36}$Cl efflux, sodium chloride efflux; CAMP, cyclic adenosine monophosphate.
known to be affected by CF in vivo) have alternate mechanisms for chloride transport.

Eccrine sweat glands are organs that are relatively easy to obtain, can be cultured (26-29), and are affected in the disease. The glands consist of a continuous tube functionally and structurally divided into a secretory (coil) region and a reabsorptive (ductal) region. Both parts are affected in CF: impermeability to chloride ion has been shown in the duct (4-6), and lack of response of sweat secretion to β-adrenergic stimuli has been found in coil (30). Recently, it was shown that primary cultures of sweat duct (31-33) and secretory coil (33-34) retain their basic electrophysiologic properties. We undertook studies of 36Cl efflux in cultured secretory cells in order to develop a cell culture model that might be useful for further investigations of the biochemical defect.

MATERIALS AND METHODS

Reagents—Collagenase CLS2, 188 units/mg, was from Worthington. MCDB 170 basal medium (35) was from the Cell Culture Facility of the University of California, San Francisco. Insulin and transferrin were from Collaborative Research. Human recombinant epidermal growth factor was from Amgen. Bovine pituitary extract was from Hammond Cell Tech. Fetal bovine serum was from Irvine Scientific; Fetal bovine serum was from Collaborative Research. Human recombinant epidermal growth factor, DPH and anti-epidermal growth factor were purchased from Sigma.

Cell Culture—Dermatologic punch biopsies, 3 mm in diameter, were taken from the forearm of CF and control volunteers, 18-36 years old, after informed consent had been obtained. The control volunteers were healthy individuals who had no family history of cystic fibrosis. Culture of sweat gland cells was performed essentially as described by Collie et al. (27). Briefly, the skin samples were incubated overnight in MCDB 170 medium containing supplements, 0.2% bovine serum, 0.4% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Following this treatment with collagenase, sweat glands were dissected out of the skin and small explants of secretory coil were plated onto 35-mm culture dishes (Corning) in the presence of serum-free MCDB 170 medium containing supplements and antibiotics (27). Typically, 15-20 plates were obtained per biopsy. Cell outgrowths appeared in 1-2 weeks. The cultures became confluent in 3-5 weeks, and survived for up to 5 months. They did not grow well after attempted subculture.

The cells that grew out of the secretory coil explants had the typical cobblestone appearance of epithelial cells and were found by electron microscopy to have structures typically found in epithelial cells: keratin filaments, microvilli, and desmosomes. The cells stained specifically for keratin 18, a marker for glandular epithelial cells (36). The cultures frequently formed "domes" as is characteristic of actively secreting cultured epithelial cells (29, 37), and as has been found to occur in cultures of sweat gland secretory coil (27) and ductal (29) cells. There was no difference in cell morphology or growth pattern between CF and control cells. Frequently, two kinds of cells were observed in individual plates, large and small; both were judged to be epithelial by the above criteria and may represent the granular and agranular cells which are known to comprise the secretory coil in vivo (36). The larger cells grew slowly and tended to die out. Experiments described below were performed on cultures containing small cells only; the appearance of these cells by light microscopy was as described by others (27, 35).

36Cl Efflux—Efflux was measured in confluent primary cultures of secretory coil cells by a modification of the procedure described by Pato et al. (39). All steps were performed in ambient air at 37 °C unless otherwise specified. The cell layer in each dish was rinsed with 2 ml of Hanks balanced salt solution (HBSS), 30% NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 5.5 mM glucose, 1.0 mM NaH₂PO₄, 0.8 mM MgSO₄, pH 7.4) before incubation in 1 ml of the buffer containing 10 μCi of NaCl. After 40-45 min (preliminary experiments showed that accumulation of 36Cl by the cells had leveled off in that period), buffer was removed and the layer was washed by submerging the dish in two consecutive 300-mll baths of 0.25% sucrose. The sucrose was aspirated and 2.2 ml of buffer (same as above) was added to the dish on a vigorously rotating platform. Aliquots of buffer (0.1 ml) were removed at specified times for measurement of radioactivity by scintillation spectrometry; the times selected were 5, 20, 40, 60, 80, 100, and 120 s after the start of the chase and less frequently thereafter. Activators or inhibitors, if any, were present in the last 10 min of labeling and throughout the chase. At the end of the experiment, cells were solubilized in 0.1% sodium dodecyl sulfate for determination of cell-associated radioactivity and measurement of protein (40).

Calculations—The amount of cell-associated 36Cl at any given time was determined by subtracting the radioactivity that was harvested into the buffer between each set of consecutive time points. A correction was made at each point for the aliquot removed for scintillation counting. The zero point was determined by extrapolation after the curve was computer-fit to an equation for double exponential decay. This protocol was followed because the medium contained, in addition to 36Cl released from the cells, a variable amount of contaminating radioactivity from the labeling period. The equation used was Y = Ae⁻ᵏᵃ + Be⁻ᵏᵇ, where Y is the amount of intracellular 36Cl at time t, A is the contribution to efflux of the fast component, B is the contribution of the slow component, and kₐ and kₗ are rate constants. Statistical analysis was performed by Dr. Jeffrey Gornbein of the Statistical/Biometric Consulting Service. Analysis of variance (ANOVA) was performed with the program SAS (SAS Institute, Cary, NC) to compare median parameters.

RESULTS

36Cl efflux was measured in primary secretory coil cultures that had reached confluence (4-19 weeks in culture). Because the concentration of extracellular chloride remained constant, the kinetics represent equilibration of the label into a large extracellular pool. At 37 °C, the release of 36Cl from control cells was biphasic and consisted of a fast and slow component (Fig. 1). Both components were almost completely inhibited at low temperature, indicating an active metabolic process. The fast component was inhibited when efflux was carried out in the presence of the apical anion channel blocker, DPC, 1 mM (Fig. 1). Other compounds that can block anion channels, DIDS (0.1 mM) and 9-anthracene carboxylate (7 mM), inhibited the fast component to the same extent, while furosemide (1 mM), an inhibitor of Na/K/Cl cotransport, had no effect on efflux (data not shown).

36Cl release from CF cells was also biphasic, and the response to inhibitors was the same as for control cells (data not shown). However, a difference in the magnitude of the fast component was detectable in results obtained from 23 control efflux experiments (cultures derived from 13 individuals) and 16 CF efflux experiments (cultures derived from 6 individuals) as summarized in Fig. 2. The kinetic parameters obtained in the individual experiments, A, kₐ, and kₗ, were subjected to statistical analysis (B was not included, since it

**FIG. 1.** Effect of DPC and cold on 36Cl efflux from control secretory coil cells. Cells derived from a control individual were grown 8 weeks in culture. 36Cl efflux was performed at 37 °C as described under "Materials and Methods" in the absence (●) or presence (▲) of 1 mM DPC. For the 6 °C study (▲), the cells were labeled as described then submerged in chilled sucrose; the chase was performed in the cold.
Kinetic parameters of $^{36}$Cl efflux from control and CF secretory coil cells at 37 °C

Median parameters, compiled from the experiments described in Fig. 2, were analyzed for statistical significance. $B$, which is defined as $1 - A$, was not included in the analysis. $k_f$ and $k_b$ are reported as (min$^{-1}$).

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**DISCUSSION**

We have shown a difference in the release of chloride ion from control and CF cells cultured from the secretory coil of sweat glands. At 23 °C, the efflux of $^{36}$Cl was similar between cultures of the two genotypes. However, efflux of $^{36}$Cl from control cells could be stimulated by the presence of isoproterenol or cAMP analogs, whereas efflux from CF cells was not stimulated by these $\beta$-adrenergic agents. At 37 °C, the efflux from control cells was faster than from CF cells but could not be stimulated further. The similar yet nonadditive effects of temperature and $\beta$-adrenergic agents on control cells suggests that metabolites present at 37 °C perform the same function as the exogenous activators at 23 °C. By the same token, chloride efflux from CF cells, which is resistant to stimulation by $\beta$-adrenergic agents at 23 °C, would also be resistant to stimulation by endogenous activators at the higher temperature. These results are in accordance with electrophysiological studies which have demonstrated decreased chloride permeability across the cell membrane and lack of $\beta$-adrenergic stimulation of chloride channels in CF cells and tissues (4-19).

Efflux of $^{36}$Cl was found to be biphasic and the experimental data could be described by the equation $Y = A_0 e^{-kt} + B e^{-kt}$. Inhibition by 9-anthracene carboxylate, DPC, DIDS, all of which block anion channels, affected the fast component, $A_0 e^{-kt}$, suggesting that this component represents efflux through chloride channels, whereas the slow component, $B e^{-kt}$, represents release by some other mechanism. While the equation assumes two pools of $^{36}$Cl, A and B, from which efflux proceeds at different rates, it does not specify the physical nature or location of these pools. Three possibilities come to mind: 1) A represents a cytosolic pool of $^{36}$Cl and B an extracellular pool; 2) A represents a cytosolic pool and B a pool within membrane-bound organelles; 3) both A and B represent cytosolic pools located within cells that are in different metabolic states.

The almost complete absence of efflux in the cold indicates that both the fast and slow components of efflux are mediated...
by metabolic processes, and rules out the first possibility, which envisages a slow release from some pool of bound extracellular $^{36}$Cl. A consideration of the effects of temperature and activators on the kinetics of efflux from control cells may allow us to discriminate between the other alternatives. In the second hypothesis proposed above, the fast component of efflux would be cytosolic $^{36}$Cl leaving through channels in the plasma membrane and the slow component would be organellar $^{36}$Cl that first had to cross an organelar membrane by some rate-limiting mechanism. The opening of channels in the plasma membrane by $\beta$-adrenergic agents might be expected to increase the rate constant of the fast component, $k$, but not the magnitude of the cytosolic pool $A$. This prediction does not fit the data, which shows that $A$ is the only parameter to change. The third hypothesis would have cells be in an "activated" state (chloride channels open, efflux rapid) or "not activated" (channels closed, efflux slow). In CF cultures under all conditions, and in control cultures at 23 °C, only a small fraction of the cells would be in an activated state at any given time and the A pool would be small. Upon stimulation by temperature or $\beta$-adrenergic agents, there would be a severalfold increase in the number of control cells achieving the activated state. This would result in an increase in the fast component of efflux without a change in the rate constant, in keeping with the experimental data.

The results show cultured sweat gland secretory cells display a "CF phenotype" that can be observed by methods other than electrophysiology. However, the system described above has limitations because the cells could not be subcultured. With primary cultures, the total number of experiments that can be performed on cells of any one individual is small, and experiments must be conducted on cultures that are not identical sister plates. The system may prove useful when applied to immortalized CF and control epithelial cell lines, which are now becoming available (41, 42). Good cultured cell models should be particularly valuable for testing the expression of the cystic fibrosis gene.

Acknowledgments—We thank Guilda Zokaeem and Doris Quon for their help in setting up the culture system, Dr. Jeff Gornbein (Department of Anatomy and Cell Biology) for performing the biopsies, Monica Eisinger (Department of Anatomy and Cell Biology) for performing electron microscopy, Drs. Cindy Bell and Paul Quinton (University of California, Riverside) for critical reading of the manuscript. We are grateful to all the individuals who participated in this work by donating skin biopsies.

REFERENCES