Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 233-239

KINETIC ANALYSIS OF CHLORIDE EFFLUX FROM NORMAL AND CYSTIC FIBROSIS FIBROBLASTS

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Received November 28, 1985

Chloride permeability in 9 cystic fibrosis- and 11 normal-skin fibroblast lines has been investigated. Chloride efflux, under steady-state conditions, involves two intracellular compartments characterized by slow- and fast-rate constants of efflux. We show here that the fast rate constant in cystic fibrosis cells is reduced by 25% in comparison with controls. The data presented support recent studies indicating that isolated sweat glands and respiratory epithelia of patients suffering from cystic fibrosis have an unusual low permeability to chloride ions compared to control epithelia. It is concluded that variation in chloride transport can successfully be studied in cultured fibroblasts, which are not directly involved in the pathology of the disease.

Cystic fibrosis (CF) is a inherited genetic disease of the exocrine glands which is characterized by a generalized epithelial dysfunction involving an alteration in fluid and elecrolyte transport. The molecular basis of this disorder is still unknown, although in the past several metabolic abnormalities have been reported (for a review see 1). Recently, significant contributions in the study of CF have been obtained through use of measurements of the luminal potential differences in sweat duct (2) and respiratory (3) epithelia. These elecrophysiological studies have shown the existence of a marked decrease in chloride permeability across the epithelia of CF patients. It has been proposed that under these circumstances, the transport of NaCl across epithelia should be greatly impaired and this would account for the raised elecrolyte level in patients' secretion. This tentative conclusion, although suggesting some biochemical insights into the pathology of the disease, does not clarify the intimate mechanism of chloride permeability. Indeed, because of the particular

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cellular architecture of epithelial cells, characterized by the presence of luminal and basolateral membranes, it is a priori difficult to establish both location and molecular mechanism leading to an altered chloride permeability. Owing to this, we have sought to determine whether a different cellular system such as cultured skin fibroblasts, even if not involved in the pathology of the disease, might be successfully used to identify alterations in membrane chloride permeability. The data presented strongly indicate that cystic fibrosis fibroblasts are indeed impaired in chloride permeability.

MATERIALS AND METHODS

Cells cultures : skin fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and antibiotics (penicillin 100 UI/ml; streptomycin 100 ug/ml) in a humidified athmosphere of 5% CO_2 in 95% air at 37°C. Cells were removed from stock flasks by trypsinization and prepared for the experiments by seeding approximately 4×10^4 cells into 2 cm^2 well of a Nunc 24 wells cluster dish. Cells were allowed to grow to confluency in 1 ml of growth medium for 7 days.
Cloride efflux : the growth medium was removed by aspiration and cells were washed twice with the incubation medium containing 130mM NaCl, 3mM KCl, 1mM NaH_2PO_4, 1.3mM $CaCl_2$, 1.5mM $MgCl_2$, 10 mM D-glucose and 20mM Na-HEPES pH 7.4. After washing, cells were preincubated with 0.2 ml/well of the incubation medium containing 7 uCi/ml of $Na^{36}Cl$ at 37°C. After 1 hour, $^{36}Cl^-$ equilibration was stopped by removing the radioactive medium and washing the cells twice with 2ml/well of cold 0.25M sucrose and 5mM Na-HEPES. The rinse procedure was performed as described in (4) and took less than 15 seconds. Cells were then incubated with 0.3 ml/well of the incubation medium without $^{36}Cl^-$. At defined times the efflux medium was removed and cells solubilized with 0.3 ml/well of 0.2 M NaOH, kept for 10 min at 55°C. Aliquots of cell lysate were taken and added to 5 ml of MP Beckman scintillation fluid containing 0.7% acetic acid and counted for radioactivity with a Beckman LS 1800 liquid scintillation spectrometer. Protein content was determined according to Lowry et al. (5) in aliquots of cell lysate.
Materials : Foetal calf serum, growth medium and antibiotics were purchased from Gibco. $Na^{36}Cl$ was purchased from Amersham, DIDS from Sigma.

RESULTS

In the present study chloride permeability in cystic fibrosis and normal fibroblast lines has been analyzed following $^{36}Cl^-$ efflux under steady-state conditions. The curve in Figure 1 represents the efflux of Cl^- from normal fibroblasts. The measured efflux process consists of a superposition of two efflux events (operationally defined as two compartments), each exhibiting first order exponential decay. According to (6), the amount of

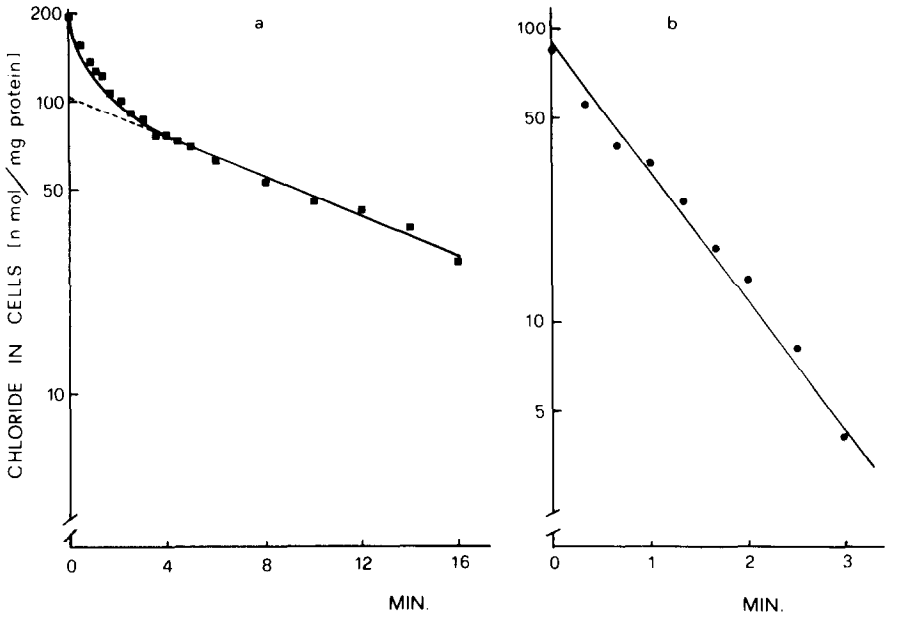

Fig. 1 : Steady-state chloride efflux from human fibroblasts. a) Kinetics of total $^{36}Cl^-$ efflux. To obtain the rate constant for efflux from the fast compartment, the extrapolated efflux from the slow compartment was substracted from the total chloride efflux and the differences replotted in b).

tracer ions present within the cells at time t, X(t), can be expressed as follows:

$$X(t) = A\ e^{-K_A t} + B\ e^{-K_B t}$$

where A and B are the amplitudes of the compartments A and B respectively and K_A and K_B are the rate constants of efflux from the respective compartments. As previously shown in (7) the two compartments can be resolved graphically assuming that the efflux from compartment A relative to total efflux becomes negligible at times longer than 3 minutes, (figure 1 a); back-extrapolation of this curve to time zero yields the effective amplitude (nmoles/mg protein) for compartment B (slow compartment). The value of K_B can be calculated from the half-life of this compartment. The amplitude and K_A of compartment A have been determined by subtraction of the extrapolated efflux due to the slow compartment from the total chloride efflux. The differences have been replotted in Figure 1b, which represents the contribution from compartment A (fast compartment).
The experiment described in Figure 1 indicates a compartment amplitude of 90 nmoles/mg protein and 102 nmoles/mg protein for A and B respectively

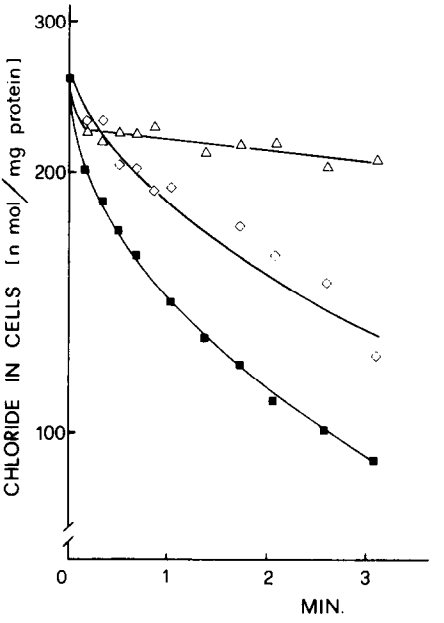

Fig. 2 : Effect of low temperature and DIDS on chloride efflux. Cells were treated exactly as described in fig. 1a: efflux was performed at 37°C (■) and at 2°C (◇); efflux medium containing 50 μM DIDS (△) at 37°C.

and a K_A of 1.040 min^{-1} and K_B of 0.079 min^{-1}. The line drawn through the points represents the best fit for the experimental points determined by a computer program based on standard non-linear least squares minimization procedure (8).

In Figure 2 it is shown that the fast phase of $^{36}Cl^-$ efflux is drastically slowed down at low temperature; the slow phase is also partially inhibited (not shown). Figure 2 also shows the effect of DIDS (4,4'-diisothiocyanostilbene 2,2'-disulfonic acid) on $^{36}Cl^-$ efflux from fibroblasts. DIDS is a specific non-permeant inhibitor of anion exchange in red blood cells (9) and in Ehrlich ascites cells (10). It is apparent that DIDS almost completely abolishes the fast phase of $^{36}Cl^-$ efflux, along with a substantial reduction of the slow phase (not shown). Using the experimental procedure adopted in Figure 1, we determined $^{36}Cl^-$ efflux from 9 CF lines and 11 control lines. In figure 3 A and B, the rate constants of efflux from the two compartments in normal and CF cells have been compared. Each experimental point represents the average of at least two, normally three, experiments performed on the same cellular line. The average value for K_A is decreased by 25% in CF cells ($p < 0.001$), while the average value for K_B does not differ significantly

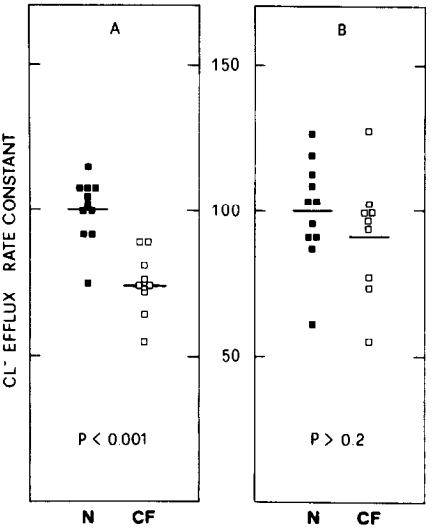

Fig. 3 : Chloride efflux in normal (N) and CF fibroblasts. The values of K_A and K_B for chloride efflux were calculated as described in fig. 1 and expressed as percent of average values found in all the control cells. A) the average value for K_A was 1.033 min $^{-1}$; B) the average value for K_B was 0.075 min $^{-1}$. 6 CF lines and 10 control lines were assayed in a double blind procedure. Statistical evaluation was based on the Student's t test.

from normal (p 0.4). Evaluations of compartment amplitudes were obtained by the y intercept in the semilogarithmic plot, as described in Figure 1, assuming that they are arranged in parallel. No significant difference has been seen in both compartment amplitudes and total cellular chloride concentrations (not shown).

DISCUSSION

In this study we have conclusively demonstrated that Cl^- efflux from normal fibroblasts involves two intracellular compartments characterized by slow and fast rate constants of efflux. In this respect, it is noteworthy that the values of the rate constant of efflux from the slow compartment are very similar to those previously reported by Pato et al. (6) in a similar cellular system, whereas the values of the fast rate are slightly higher in our experiments. This discrepancy might be simply due to the different experimental procedures employed.

One can postulate that fast- and slow- compartments may represent subpopulations of cells at different growing phase. This possibility however is unlikely because all the experiments were performed in highly confluent cultures, containing a predominance of resting cells.

Furthermore, the alternative explanation that one of the compartments, mainly that linked to the fast-rate efflux, could be associated with an extracellular phase can be ruled out by experiments of Figure 2 showing that Cl^- efflux is strongly affected by low temperature. This clearly indicates that both compartments are intracellular, while the initial temperature-insensitive loss of radioactivity which is also apparent in Figure 2 is likely to represent wash-out of $^{36}Cl^-$ from extracellular compartments of cells.

Our observation that a non-permeant inhibitor such as DIDS almost completely abolishes the efflux from the fast compartmartment (shown in figure 1) is of particular interest since it might indicate chloride transport across the plasma membrane. If this assumption is correct, it follows that the fast rate component corresponds to the cytoplasmic compartment of fibroblasts.

At present, the identity of the slow compartment is unknown. One possibility is that it could be the membrane -limited compartment of a subcellular organelle as previously suggested (6), since no direct evidence for a bound cytoplasmic Cl^- pool has been found in cellular systems studied to date (11,12).

From the data reported in Figure 3, it can be concluded that Cl^- efflux associated with transport across the plasma membrane of CF fibroblasts is significantly reduced in comparison to controls. The abnormality in chloride transport can therefore be successfully detected in a cell system as cultured skin fibroblasts which are not directly involved in the pathology of the disease. A slight discrepancy, however, exists between the data shown here and those reported in respiratory epithelia (3) in which chloride fluxes in CF were 50% reduced in comparison with normal tissue, this latter value including a 26% reduction in permeability of mannitol, a marker of solute flow through the paracellular path.

In summary, although we are concerned that the effect we have herein reported on chloride permeability might be the result of multiple biochemical phenomena, we feel that the difference observed in the kinetic behaviour between control and CF fibroblasts can be an important lead in the search of the basic defect of this disorder.

ACKNOWLEDGEMENTS

We wish to than Prof. F.A.Bonne', Paris, and Dr.M.Rocchi, Institute Gaslini, Genoa, for kindly providing fibroblasts lines and Dr. L.Prosperi and Dr. M.Battino for growing cell cultures. Prof. D.Zannoni is thanked for helpful discussion and critically reading the manuscript.
This work was supported by grants from the "Progetto Finalizzato: Ingegneria Genetica e Basi Molecolari delle Malattie Ereditarie", C.N.R., Rome.

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