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Cl^- Permeabilities in Red Blood Cells and Peripheral Blood Lymphocytes from Cystic Fibrosis and Control Subjects

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ABSTRACT. Recent studies have identified abnormalities in Cl^- permeation across two target cystic fibrosis (CF) epithelia (sweat duct and respiratory epithelium). In the present study, anion conductances of red blood cells (RBCs) and peripheral blood lymphocytes (PBLs) from CF and normal subjects were estimated and compared. For RBCs, the valinomycin-induced rate constant for K^+ loss (P_{K^+}) was taken as an index of P_{Cl^-}. For PBLs, the secondary volume increase after gramicidin pretreatment and hypotonic (0.67× isotonic) stress was used to estimate P_{Cl^-}. The Cl^- permeabilities of RBCs and PBLs from CF and control subjects were comparable. These findings suggest that the abnormality in P_{Cl^-} reported for CF sweat ductal and respiratory epithelia is not expressed in circulating blood elements. (*Pediatr Res* 18:1336–1339, 1984)

Abbreviations

CF, cystic fibrosis
PBL, peripheral blood lymphocyte
RBC, red blood cell
P_{Cl^-}, Cl^- permeability coefficient
P_{K^+}, K^+ permeability coefficient
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DIDS, 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid
SITS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid

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Cystic fibrosis is a disease that is characterized by multiple abnormalities in the volume and composition of body "secretions" (5). This feature has led many investigators to suggest that the disease may reflect a generalized dysfunction in ion permeation (5). Evidence has accrued that the CF sweat ductal epithelium may be relatively impermeable to Cl^- ions (23, 26). A reduction in cell Cl^- permeability also appears to be a characteristic of CF respiratory epithelia (17, 18). The defect at each site appears to involve a path for Cl^- that is electrically conductive. It is not yet known whether this dysfunction reflects a structural or functional (control) abnormality.

Techniques have recently been developed to identify and partition Cl^- movements across membranes of circulating blood elements to either conductive or nonconductive paths. In the RBC, ion and water movement across valinomycin-treated cells reflect conductive Cl^- flows (13, 14). In the PBL, volume regulatory changes in response to an isosmotic medium have been used to study conductive paths for K^+ and Cl^- (9, 10). Because an abnormality in Cl^- channels might be generalized to blood elements in CF subjects, we compared the magnitude and regulation of conductive Cl^- paths in RBCs and PBLs from CF subjects with those from control (normal) subjects.

MATERIALS AND METHODS

Subjects. Blood was obtained in heparinized tubes and used for study within 4 h of venipuncture. Blood was obtained from

three males and three females with cystic fibrosis. These patients ranged in age from 16 to 45 years of age, and all were characterized by typical clinical criteria, raised sweat Cl^- values, and raised nasal potential differences (17). Four subjects were taking antibiotics of the penicillin class at time of study. No subjects were taking diuretics, cardiac glycosides, methylxanthines, or β-agonists. All control subjects (ages 21–46, two females, four males) were free of disease, were not taking medication, and had negative family histories for cystic fibrosis. All research procedures were performed in accordance with procedures approved by the UNC Committee on Human Experimentation and informed consent was obtained.

Measurement of Red Cell Cl^- Permeability. Red cells from heparinized blood were washed three times in a medium that contained (mM): NaCl (130), KCl (1), HEPES (10) (pH 7.4 at either 25° C or 37° C). The medium also contained absolute ethanol (0.25 ml/liter). Washed cells were suspended in 15 volumes of this medium at the desired temperature, and a sample was removed for the "zero time" determination of Na^+, K^+, and water content. Valinomycin (22.5 mM stock in absolute ethanol) was then added (final concentration, 10 μM). The concentration was twice that needed to induce maximum K^+ efflux. At 5, 10, 20, and 30 min after addition of the drug, samples were removed from the red cell suspension and centrifuged at 4° C for determination of cell ion and water contents. A graph was plotted on semilog paper of cell K^+ (in mmol/kg dry cell weight) as a function of time in minutes. The slope of the relationship [$\ln (K_t/(K_{zero})]/t$ is the rate coefficient (P'_{Cl}) in min^{-1} which was equated to P_{Cl}.[1] Procedures for the measurement of cell ions and water have been published previously (21).

Measurement of PBL Cl^- Permeability. Mononuclear cell preparation. A mononuclear cell fraction was separated from peripheral blood by the method of Grinstein *et al.* (9). Briefly, heparinized whole blood was diluted 50:50 with RPMI 1640 tissue culture medium and then layered over Ficoll-Hypaque (Histopaque 1077, Sigma Chemicals). After centrifugation, the mononuclear cells from the interface were washed in phosphate-buffered saline and then examined and counted under phase contrast microscopy in a hemocytometer chamber. Trypan blue staining revealed greater than 95% viability. Cells were diluted into the appropriate test solutions at a final concentration of $2–4 \times 10^4$/ml.

Cell sizing. Cell volume was measured by using the electronic aperture impedance method (Coulter principle). A suspension of cells was passed through a Coulter-type aperture (100×100 μm). A constant current source, preamplifier and pulse height analysis computer (ND-60, Nuclear-Data, Schraumsburg, IL) were used to collect pulses and display them as a volume frequency distribution. Polystyrene beads were used to calibrate and quality control the system.

Protocol. In preliminary studies, PBLs were exposed to a hypotonic stimulus by the addition of distilled water to the Krebs-Ringer incubation medium (9). Samples of PBLs were sized before and, serially, for 20 min after dilution. In the second series of experiments, PBLs were harvested and incubated in a Cl^- replete (with Krebs-Ringer medium) or Cl^- free (replaced by gluconate) Ringer's solution and cell volume was measured. Then, gramicidin D (5×10^{-5} M, Sigma) was added, and volumes were measured serially on samples of cells for 10 min. Next, the solutions were then diluted to 0.67× isotonic with distilled water and volume of cells in serial samples was followed for another 10 min.

Statistics. All values represent the mean ± SEM. Regression lines were fit to the points by least squares analysis.

RESULTS

The results of the determinations of RBC ion and water content and P'_{Cl^-} are summarized in Table 1. As noted by others (2), there were no significant differences between the cation and water content of untreated RBCs from CF subjects and those from normal subjects. The values for P'_{Cl^-} of cells from CF and control subjects were not different at 25 or 37° C. These values agree with those reported previously by Hunter for normal

Table 1. *Apparent red cell chloride permeability (P_{Cl^-}) in three patients with cystic fibrosis and three normal controls**

	Control	Patient
Control J. P.; patient P. S.		
Baseline red cell contents		
Na	14.3	9.6
K	84.8	85.6
Water	65.6	65.3
P_{Cl} at 25° C	0.013	0.015
Control R. B.; patient L. P.		
Baseline red cell contents		
Na	11.5	8.5
K	78.6	89.1
Water	64.99	67.7
P_{Cl} at 25° C	0.011	0.014
Control M. K.; patient M. C.		
Baseline red cell contents		
Na	11.6	9.8
K	80.3	84.7
Water	65.6	65.3
P_{Cl} at 37° C	0.048	0.043
Hunter's values for P'_{Cl} (14)		
25° C	0.015	
37° C	0.040	

* Values for Na^+ and K^+ are given in mmol/liter cells; water is given as per cent wet weight. P'_{Cl} is in min^{-1}.

[1] When cells in a low K^+ medium are treated with the K^+ ionophore valinomycin, they lose K^+, Cl^-, and water. During this transient, the net flux of K^+, J_{K+}, is equal to the net flux of Cl^-, J_{Cl-}. If the valinomycin concentration is so high that raising it further causes no increase in J_{K+}, then J_{K+} is limited by the rate of Cl efflux and J_K can be used as an index of P_{Cl-}, the chloride permeability. The constant field flux equation (12) predicts the chloride flux in such an experiment.

$$J_{Cl} (=J_K) = P_{Cl} \ln B \frac{[Cl_i B - Cl_o]}{B - 1} \quad (1)$$

where

$$B = \exp[-FEm/RT] = \frac{(P_K/P_{Cl})K_i + Cl_o}{(P_K/P_{Cl})K_o + Cl_i} \quad (2)$$

J_{Cl-} and J_{K+} are in mmol/kg dry cell weight/min; K^+i, K^+o, Cl^-o are the internal and external K^+ and Cl^- concentrations in mmol/liter cell water; the K^+ and Cl^- permeabilities, P_{K+} and P_{Cl-} are in $cm \cdot s^{-1}$; *Em* is the membrane potential; and *F*, *R*, and *T* have their usual meanings.

In the fresh, untreated red cell P_{K+}/P_{Cl-} is of the order of 0.01, but as the cells are exposed to increasing concentrations of valinomycin, P_{K+}/P_{Cl-} exceeds unity and rises monotonically. As P_{K+}/P_{Cl-} increases, the value for *B* in Equation 2 approaches K_i/K_o, so that at very high valinomycin concentrations the right side of Equation 1 reduces to:

$$P_{Cl} \ln(K_i/K_o) \frac{Cl_i K_i - Cl_o K_o}{K_i - K_o}$$

If the experiment is done in a dilute cell suspension, all the factors in the above expression except P_{Cl} can be regarded as constant because water moves isosmotically with K and Cl so that the concentrations of these ions in the cell do not change. Hence:

$$J_{Cl} = J_K = (\text{constant chemical driving force}) \cdot P_{Cl}$$

We found that for the first 30 min after adding a high concentration of valinomycin to the cell suspension, the loss of K from the cells described a first-order process:

$$\ln (K_t/K_{zero}) = -P'_{Cl} t \quad (3)$$

where K is cell K in mmol/kg dry cell weight at time zero (K_{zero}) or at *t* min (K_t). Since at the very high valinomycin concentrations used in these experiments $J_{Cl} = J_K$, the empirical rate constant, P'_{Cl} (min^{-1}), must express the rate of chloride loss. This constant is related to the chloride permeability, P_{Cl}, by the expression:

$$P_{Cl} = P'_{Cl}(V/S)$$

where *S* is the surface area, a constant, and *V* is the solvent volume of the cell, which in these experiments varies in direct proportion to the movements of K (and Cl). We have expressed our results in terms of the rate constant, P'_{Cl}, so that our kinetic data can be compared with those of Hunter (14).

subjects (14). Accordingly, P'_{Cl^-} in CF RBCs appears to be normal (see "Discussion").

In preliminary experiments, it was found that the volumes of CF and control PBLs in isotonic solutions were similar [214 ± 20 fl, $n = 3$; compared with 204 ± 20 fl, $n = 3$, respectively]. In addition PBLs from both CF and control subjects volume regulated when exposed to hypotonic (0.67× isotonic) solutions. The time required to return to the initial volume was similar for PBLs from each group (8.5 ± 1.0 min for CF, compared to 8.8 ± 1.1 min for normal subjects; $n = 3$ for each). Whereas this information suggests that P_{Cl^-} of PBLs from each group can increase in response to a hypotonic stimulus, a direct comparison of P_{Cl^-} between the two groups cannot be made from this protocol because P_{K^+} rather than P_{Cl^-} is rate limiting (see "Discussion").

The results of the measurements made after sequential exposure of PBLs to gramicidin and then a hypotonic stimulus are shown in Figure 1. Both CF and normal PBLs exhibited an initial volume increase in response to hypotonicity. The magnitude of the initial osmotic water uptake can be estimated by the change that accompanied exposure to a solution with Cl^- replaced by the impermeant anion, gluconate. The increase in volume is followed by a slower linear rate of swelling (Fig. 1). The rate of the secondary phase, which is directly proportional to P_{Cl^-}, is similar for PBLs from CF and control subjects. The mean rate of volume increase for the three CF subjects was 41.3 ± 4.7 fl/min ($r = 0.96 \pm 0.02$); the mean rate for controls was 40.8 ± 0.22 fl/min ($r = 0.97 \pm 0.02$).

DISCUSSION

Because blood is relatively available for study as compared to epithelia, circulating blood elements from CF subjects have been studied in some detail. The RBC in particular has been the focus of several lines of inquiry. Water permeability (7), fatty acid metabolism (25), and ATPase activity [Ca^{2+} (11, 20), Mg^{2+} (20), and Na-K^+ (11)] have been compared in CF and control RBCs. Red cell cation permeability, *e.g.* ^{22}Na influx and ouabain-insensitive efflux, have been measured to explore the notion that CF reflects a basic dysfunction in Na^+ permeability (2, 6, 19) or as an assay for pathogenic "factors" (8). Less is known about the function of CF leukocytes. Ca^{2+} uptake (1) and the β-receptor-adenylate cyclase system have been examined (4). In general, it has been difficult to determine whether the differences noted in CF cells reflect primary defects or sequellae of chronic disease.

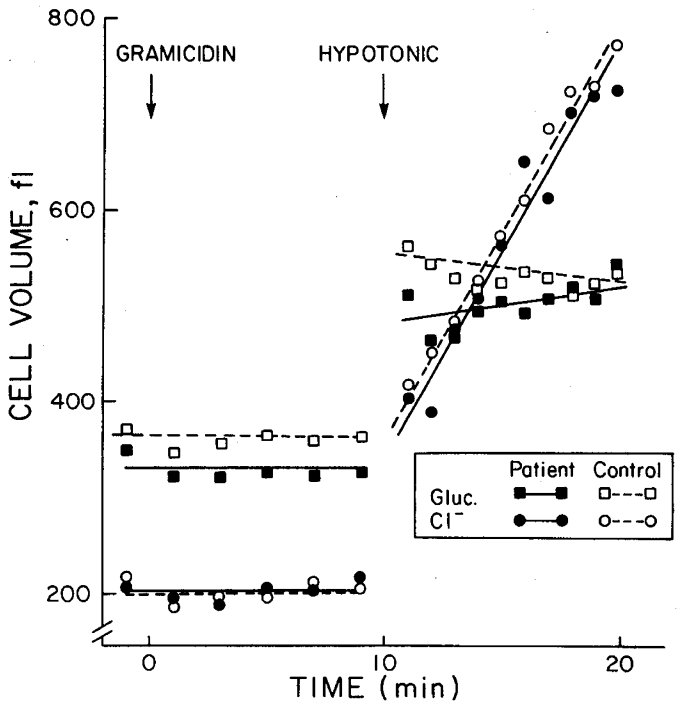

Fig. 1. Peripheral blood lymphocyte volumes for CF and control subjects as a function of time. *Circles* indicate PBL volume measurements made in Cl^- replete Ringer's solution. *Squares* indicate PBL volume measurements made in Cl^- free [replaced by gluconate (*Gluc.*)] media. *Arrows* indicate sequential additions of gramicidin (5×10^{-5} M) and hypotonic stimulus (0.67× isotonic). Values shown are the mean of measurements for three subjects/group. SEM equal to or less than 5% at each *point*. *Lines* depict least squares regressions for times before and after hypotonic stimulus.

The present studies were designed to characterize anion flows across RBCs and PBLs from CF and normal subjects. The rationale for the study was the recent identification of a defect in conductive Cl^- permeation across at least two epithelia that are affected by CF (17, 18, 23, 26). Our study was facilitated by reports of others (9, 10, 13–15) of strategies that permit the separation and accurate measurement of conductive Cl^- permeability of certain circulatory cells.

Net Cl^- flow (which is the same as Cl conductance) was measured by a method adapted from the work of Hunter (13, 14) and Knauf *et al.* (15). The values of Cl^- flow, expressed in units of P'_{Cl^-}, during valinomycin exposure for the normal subjects are similar to those previously reported (Table 1). Moreover, P'_{Cl^-} is not different for RBCs from CF as compared with control subjects during the period valinomycin induced KCl and water loss. It is possible that P'_{Cl^-} in the basal state (before the addition of valinomycin) is not changed by the addition of the ionophore, and, hence, the value for P'_{Cl^-} represents basal P'_{Cl^-}, but this notion cannot be rigorously tested. Consequently, we conclude that the P'_{Cl^-} estimated from KCl loss induced by valinomycin is similar for CF and normal erythrocytes and may be similar in the basal state.

The process of volume regulation by the lymphocyte has been reviewed recently by Grinstein and co-workers (10). Important features of the lymphocyte membrane include: 1) a conductive path for K^+ (P_{K^+}) that is greater at rest, than the conductive path for Cl^- (P_{Cl^-}); 2) the cells respond to swelling in hypotonic media, with increases in both P_{K^+} and P_{Cl^-} and a loss of KCl and water; 3) conductive K^+ and Cl^- flows during volume regulation appear to be independent; and 4) the Ca^{2+} ionophore, A23187, appears to increase both P_{K^+} and P_{Cl^-}, suggesting a role for Ca^{2+} in the membrane response to hypotonicity.

The lymphocytes from CF subjects and normal controls volume regulated to a basal volume over a similar time course during exposure to a hypotonic stimulus (0.67× isotonic), suggesting that both P_{K^+} and P_{Cl^-} increased to permit KCl and water loss. Because P_{K^+} rather than P_{Cl^-} is rate limiting during volume regulation (9, 10), these findings imply that P_{K^+} is similar in both groups but quantitative comparisons of P_{Cl^-} between the two groups is not possible.

The experiments depicted in Figure 1 permit a comparison of the magnitude P_{Cl^-} in CF and control PBL during volume regulation. When gramicidin was added to isotonic medium (Cl^- free or Cl^- replete), little change in the volume in CF or control PBLs was noted. Since gramicidin greatly increases cation conductance, this finding suggests that P_{Cl^-} is small and rate limiting in PBLs from both populations.

During exposure to hypotonic media, PBLs from each population that were incubated in either Cl^- containing or a Cl^- free media (gluconate) exhibited a rapid increase in volume. This increase reflects rapid redistribution of water. However, because gramicidin pretreatment in Ringer's solution ($K^+ = 5$ mM) loads the PBL with Na^+ and depletes K^+, there is no gradient for cation loss. Instead, there is a gradient for Cl^- entry into PBLs that are incubated in Cl^- replete media. It is this gradient for anion entry which supports the secondary phase of PBL swelling. Because the anion gradient induces swelling, and because cation conductance is nonlimiting in the presence of gramicidin, the rate of secondary swelling is directly proportional to P_{Cl^-}. This notion

is experimentally confirmed by the absence of a secondary phase when the impermeant anion gluconate is substituted for Cl^-. Thus, comparison of the slopes of the secondary volume changes should give an estimate of P_{Cl^-}. The slopes for the two populations are indistinguishable, suggesting that a normal increase in P_{Cl^-} of PBLs from CF patients is induced by osmotic swelling.

Our studies fail to show that CF affects P_{Cl^-} in RBCs or the control of P_{Cl^-} in PBLs. There are two obvious explanations for the normal Cl^- permeability of circulating blood elements from CF subjects and the derangement in Cl^- permeability described for two target epithelia. First, the Cl^- paths may be structurally different in epithelia and circulating blood cells so that a generalized dysfunction would not be expected. Conductive Cl^- flow across the RBC appears to involve the band III anion exchanger. Whereas conductive flow through this path is many times ($\sim 10^4$) smaller than exchange (Cl^--Cl^-, or Cl^--HCO^-_3) flow, both paths are DIDS or SITS sensitive (18). In contrast, Cl^- flow across the PBL membrane is DIDS insensitive, suggesting that even conductive Cl^- of circulating blood elements may be different. The DIDS sensitivity of the conductive Cl^- path in respiratory or sweat ductal epithelia has not been reported.

Alternatively, the regulatory pathways that control membrane anion conductances may be different in epithelia and circulating blood elements. Again, a detailed comparison of the control of Cl^- flows across barriers in epithelia and blood cells is difficult because of important gaps in pertinent information about both tissues. Recent studies in normal human nasal epithelium *in vitro* (3) have demonstrated that exposure to β-agonists, prostaglandin E_2, or A23187 increases cell Cl^- permeability. There are not comparable data for human sweat ductal epithelia. As discussed above, A23187 can increase P_{Cl^-} in PBLs but its mode of action, in these cells and in respiratory epithelia, is not known. PBLs have β-receptors and adenylate cyclase (4) but the relationship of this process to P_{Cl^-} has not been reported. Human RBCs do not have β-receptors, or adenylate cyclase, nor do they produce prostaglandins in any quantity (22, 24). A link between cell Ca^{2+} or the effects of A23187 and conductive P_{Cl^-} has not been reported. Consequently, whereas some of the possible processes that regulate P_{Cl^-} in epithelia may overlap those of blood elements, there is not enough information to make a fair comparison.

In summary, P_{Cl^-} values estimated from valinomycin-treated RBCs and hypotonically swollen PBLs from CF and control subjects are not different. Accordingly, these data suggest that a defect in Cl^- permeation is not a generalized dysfunction in CF but reflects a high degree of tissue specificity.

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