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Calcium-ATPase Activity in Cystic Fibrosis Erythrocyte Membranes: Decreased Activity in Patients with Pancreatic Insufficiency

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Summary

The activity of Ca-ATPase (Ca^{2+},Mg^{2+}-ATPase, ATP phosphohydrolase, EC 3.6.1.3) was measured in erythrocyte membrane preparations from 37 cystic fibrosis patients, 27 with pancreatic insufficiency and 10 with pancreatic sufficiency, and from 24 healthy controls. The mean maximal calcium-stimulated specific activities, in the absence and presence of purified calmodulin, of the pancreatic sufficient patients (34.3 ± 4.2 and 75.9 ± 6.9 nmol/min/mg) was indistinguishable from that of controls (35.8 ± 2.6 and 84.3 ± 4.7 nmol/min/mg), while both activities of patients with pancreatic insufficiency were significantly decreased (28.9 ± 1.3, $p < 0.02$; 65.2 ± 3.0, $p < 0.001$) compared to the control group. Similarly, the mean erythrocyte membrane (Na + K)ATPase activity was decreased only for those patients with a history of steatorrhea and who clinically required pancreatic enzyme therapy and had low immunoreactive trypsin levels (10.6 ± 0.8 *versus* control, 13.4 ± 1.1, and pancreatic sufficient patients, 13.3 ± 1.4 nmol/min/mg; $p < 0.025$). No correlation was found between any of the ATPase activities and the clinical scores of the patients, suggesting the lack of significant contribution of general clinical status to the activities of those cation transporters.

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Abbreviations

CF, cystic fibrosis
EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid

Cystic fibrosis, a genetic disease involving abnormal epithelial electrolyte transport and exocrine gland dysfunction, is clinically manifested as chronic obstructive lung disease and maldigestion. In recent years, it has become increasing apparent that intracellular calcium has important roles in the regulation of many diverse cellular processes including stimulation-secretion coupling. Since the molecular disorders of the CF exocrinopathy may be closely related to calcium function, we investigated the regulation of intracellular calcium levels in CF cells.

The levels of cytosolic free Ca^{2+} and thereby its second messenger effects appear to be regulated by protein binding and by transport out of the cytosol by three membrane systems: the plasma membrane Ca-ATPase, the mitochondria, and the endoplasmic reticulum. Total intracellular calcium has been found to be elevated in leukocytes (3), fibroblasts (42), lymphocytes (42), and parotid acinar cells (34) from CF patients. Shapiro and coworkers (17, 42) have observed an increased sequestration of calcium by mitochondria from CF fibroblasts. There has been disagreement for the past decade regarding decreased Ca-ATPase activity in CF cells (1, 7, 16, 18, 24, 27–30, 35, 47). Recently, Katz and coworkers (1, 27–30) have found the greatest difference in calcium-stimulated ATPase activity between CF and control erythrocyte and fibroblast membranes, both in the presence and absence of the activator protein calmodulin. Foder *et al.* (18), using a different technique for erythrocyte membrane preparation, have observed less dramatic decreases for CF Ca-ATPase activity.

The plasma membrane Ca-ATPase (ATP phosphohydrolase, EC 3.6.1.3, Ca^{2+},Mg^{2+}-ATPase) can be conveniently studied in isolation from mitochondria and endoplasmic reticulum in preparations of open erythrocyte ghosts. In addition to activation of the Ca-ATPase by Ca-calmodulin, recent studies on the purified enzyme in reconstituted systems (see Ref. 5) have indicated several possible sources of regulation, some of which may be of biological importance. Different methods of preparing the membranes for assay can result in different content of these regulators. This probably accounts for different observations on CF membranes from various laboratories. An additional factor is the great variability of the activity of the enzyme from one erythrocyte preparation to another even using the same method of preparation (21, 36, 47).

In this study, we repeated the experiments of Foder *et al.* (18) using current methodologies, modified to minimize experimental variabilities. In addition, we segregated the CF population into two subpopulations on the basis of clinical and laboratory assessment of pancreatic function. These subpopulations were compared to healthy controls in regards to basal Ca-ATPase activity, calmodulin-stimulated Ca-ATPase activity, and (Na + K)-ATPase activity.

MATERIALS AND METHODS

Subjects. Blood samples were drawn from three populations of subjects, healthy controls, age and sex matched to the CF subjects; CF patients, classified as pancreatic insufficient on the basis of history of steatorrhea, need for pancreatic enzyme supplementation, and low levels of plasma immunoreactive trypsin (see below); and CF patients classified as pancreatic sufficient on the basis of no history of chronic steatorrhea, lack of the need for pancreatic enzyme supplements, and plasma immunoreactive trypsin levels comparable to the control group (see "Results"). All CF patients had sweat chloride levels greater than 70 mEq/liter as determined by the Gibson-Cooke method (20) plus at least one of the major diagnostic criteria (chronic obstructive lung disease, maldigestion, sibling with CF). Patients were scored clinically by a modification (10) of the Shwachman-Kulczycki method (43). All studies were approved by the local Institutional Review Board.

Preparation of erythrocyte ghosts. The procedure used was a modification of the method of Farrance and Vincenzi (13) for preparing erythrocyte ghosts depleted of calmodulin. Ten-ml blood samples were collected in citrate-phosphate, anticoagulant buffer, stored at 4°C, and the ghosts were prepared and analyzed within 48 h. The blood was centrifuged and the cells washed 4 times with 155 mM NaCl, sequentially discarding the buffy coat; the plasma was saved for the immunoreactive trypsin determination. Approximately 4 ml of packed, washed cells were lysed with 10 volumes of ice-cold 20 mM imidazole buffer, pH 7.6, containing 0.5 mM EGTA (22 mosm) and the membranes sedimented by centrifugation for 20 min at 35,000 × *g* in a Sorvall RC-2B centrifuge. The fluffy pellet ("whitish button" was discarded) was washed 4 times with 20 mM imidazole buffer, pH 7.6, or until the membranes were free of hemoglobin. The final pellet was resuspended in 1 volume of the same imidazole buffer to which was added 0.2 mg/ml saponin (11) and assayed for ATPase activity within 4 h. Membrane protein was measured by the Lowry method (33) using bovine serum albumin as the standard.

Lysis of erythrocytes in low osmolar solutions promotes dissociation of calmodulin from the Ca-ATPase which is also dependent on a low free Ca^{2+} concentration (13). Addition of 0.5 mM EGTA to the lysis buffer in general reduced the basal, calcium-stimulated ATPase activity but not the calmodulin-stimulated, maximal activity. In our experience, the use of saponin at 0.2 mg/ml in the final suspension buffer frequently, but not always, increased both the basal and calmodulin-stimulated ATPase activity. Downes *et al.* (11) have suggested the inclusion of saponin to open any ghosts which may have spontaneously resealed and have found that this level of the detergent does not appear to directly alter the Ca-ATPase activity. The latter is also the experience of Vincenzi (46) and our own control experiments.

ATPase assay. The assay of membrane ATPase activities was also by the method of Farrance and Vincenzi (13). One hundred μl of membranes (~ 0.2 mg protein) were assayed at pH 7.1 in a 2 ml reaction mixture containing 80 mM NaCl, 30 mM KCl, 18 mM histidine, 18 mM imidazole, 4 mM $MgCl_2$, 3 mM ATP, 0.5 mM EGTA, 0.1 mM ouabain, and a free Ca^{2+} concentration of between 0.01 μM and 1 mM. Calcium-EGTA buffers at free Ca^{2+} concentrations from pCa 8.0 to 3.0 were made accordingly to a computer program (Dr. B. Lindley, Department of Physiology) which takes into account Ca^{2+} binding to EGTA, ATP, and imidazole at 37°C and pH 7.10. These buffers were checked and corrected by measurements with an Orion Ca-selectrode [calibrated by the methods of Bers (4)] under the same conditions as in the ATPase assay.

The membranes were preincubated in the assay medium at various pCa values for 20 min at 37°C in the absence of ATP. Purified bovine testicular calmodulin (see below) was added to a set of the tubes at the beginning of the preincubation to a concentration of 1.3 μg/ml (saturating under these conditions) in order to measure Ca-dependent, calmodulin stimulation of the ATPase. The reaction was begun with the addition of ATP to a final concentration of 3 mM and the tubes incubated for 60 min at 37°C in a constant temperature water bath. The reaction was stopped with the addition of 1 ml of 10% sodium dodecyl sulfate (determined to be phosphate-free). An aliquot from each tube was assayed for orthophosphate by the method of Chen *et al.* (6) in order to determine the amount of ATP hydrolysis. Identical tubes with ATP but without membranes were used to determine spontaneous ATP hydrolysis. Measurements of (Na + K)-ATPase activity were performed concomitantly in a reaction mix identical to the above with free pCa of 8.0, but in the absence of ouabain.

Purification of calmodulin. Since mammalian calmodulin is essentially invariant and since human erythrocyte calmodulin

has been shown (26) to be indistinguishable from bovine brain calmodulin, we elected to purify this ubiquitous protein from bovine testes, a tissue having a high content of calmodulin. The procedure used was adapted to bovine testes with the help of Dr. T. H. Crouch, University of Cincinnati School of Medicine, from the procedure he and coworkers have described for bovine brain (8). The final calmodulin preparation was >95% free of contaminants as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31) and ultraviolet spectral analysis with and without calcium. The purified calmodulin produced a 6–8-fold increase in the activity of cyclic nucleotide phosphodiesterase (from bovine heart, prepared by us with the method of Ho *et al.* (23); this activation was inhibited by trifluoperazine with a K_i of about 5 μM (Dearborn and Poncz, unpublished).

Immunoreactive trypsin assay. Plasma samples retained from the blood samples used for erythrocyte ghost preparations were stored at −20°C until assayed. All samples were centrifuged to remove debris prior to assay. Aliquots of 100 μl were assayed for trypsin (trypsinogen) using a Becton-Dickenson radioimmunoassay kit according to the manufacturer's directions. Control studies using other blood samples split into serum fractions and plasma fractions anticoagulated with phosphate-buffered citrate, indicated that the anticoagulant did not significantly affect the assay.

Statistical analysis. The significance of the difference of the means was assessed by unpaired Student's t test. Correlation coefficients were evaluated by linear regression, and the corresponding significance levels were determined by standard methods involving the Z' transformation of Y.

RESULTS

The characteristics of the study populations are given in Table 1. The mean clinical score of the CF patients with pancreatic sufficiency is better than the group with pancreatic insufficiency which is consistent with the observations reported by Gaskin *et al.* (19). The initial segregation of the CF patients into these two subpopulations was based on clinical criteria which were subsequently corroborated by the plasma immunoreactive trypsin levels. The control group values for plasma immunoreactive trypsin ranged from 10.4 to 48.5 ng/ml (23.2 ± 11.5 SD) and showed a positive correlation with age ($r = 0.661$; $p < 0.001$). Figure 1 depicts the plasma immunoreactive trypsin levels as a function of age for all three groups. The immunoreactive trypsin values for the CF patients classified as pancreatic sufficient (range, 9.3–88.2 ng/ml; 36.0 ± 29.7) are all comparable to or above the values for controls. When age was taken into account, the values for the pancreatic insufficient patients (range, 4.9–13.3 ng/ml; 6.3 ± 1.5 ng/ml) are all below the values of both the control and the pancreatic sufficient groups except for one patient (who did require pancreatic enzyme therapy). These results are similar to those of Durie *et al.* (12) who found an excellent correlation between serum immunoreactive trypsin levels and both duodenal aspirate evaluation of pancreatic function and fat absorption studies.

The ATPase activities of erythrocyte membranes were measured as a function of free Ca^{2+} concentration for each subject. Figure 2 illustrates a typical paired experiment comparing the ATPase activities of a control subject to those of a pancreatic insufficient CF patient. The pCa profile for Ca-ATPase was determined for both basal activity and calmodulin-stimulated

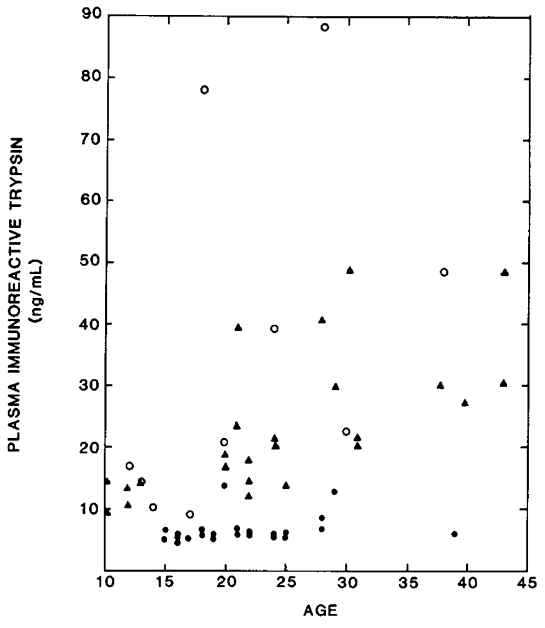

Fig. 1. Plasma immunoreactive trypsin levels as a function of subject's age. Control, ▲; cystic fibrosis with pancreatic insufficiency, ●; cystic fibrosis with pancreatic sufficiency, O.

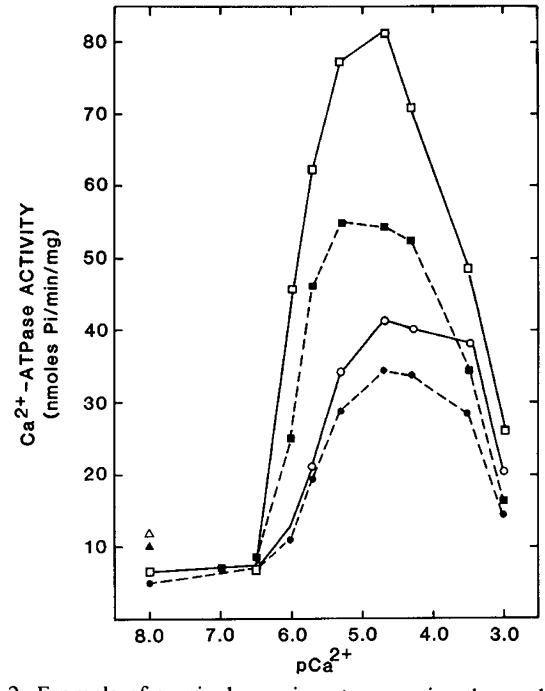

Fig. 2. Example of a paired experiment comparing the erythrocyte membrane Ca^{2+}-ATPase activities as a function of free Ca^{2+} for a cystic fibrosis patient and an age- and sex-matched healthy control. *Open symbols* and *solid lines* denote control; *closed symbols* and *broken lines* denote cystic fibrosis patient; basal, Ca-stimulated activity, O; calmodulin-stimulated activity, □; (Na + K)-ATPase activity, *i.e.* no ouabain, △.

Table 1. *Characteristics of study populations*

	n	Sex M:F	Age Mean ± SD	Age Range (yr)	Clinical score mean ± SD	Clinical score Range
Control	24	11:13	25.1 ± 9.5	10–43		
CF-pancreatic sufficient	10	8:2	21.3 ± 8.5	12–38	79.9 ± 15.0	40–90
CF-pancreatic insufficient	27	12:15	21.1 ± 5.1	15–39	63.6 ± 15.4	32–88

activity. Exogenous calmodulin purified from bovine testes was added to the calmodulin-depleted membranes to a final concentration of 1.3 μl/ml. This is well beyond saturating conditions in that half-maximal activation was observed to be approximately 91 ng/ml (5.5 nM) calmodulin and the addition of more calmodulin did not increase the maximal Ca-ATPase activity. The Mg-ATPase activity is considered to be the ATPase activity at pCa of 8.0 (with ouabain present) which, in general, was a very small fraction of the maximal ATPase activity. This activity can be eliminated by prior incubation of the membranes at 37°C (9), but in our experiments the Mg-ATPase activity was usually less than 5 nmol/min/mg protein and was therefore considered background ATPase activity and subtracted to determine the calcium-dependent ATPase activities.

The basal Ca-ATPase activity was taken as the maximal enzyme activity observed with the pCa profile in the absence of calmodulin minus the Mg-ATPase activity. The activity maximum generally occurred between pCa values of 5.0 to 4.5, with decreased activity observed at lower values of pCa. This falling off at high free Ca^{2+} concentrations, more prominent in the presence of calmodulin, has been observed by other investigators and is not totally explained but may be in part due to binding competition between Ca-ATP and the substrate Mg-ATP (9). The calmodulin-stimulated Ca-ATPase activity was taken as the maximal activity in the presence of the saturating concentration of calmodulin minus the Mg-ATPase activity. The data obtained from these experimental curves are not suitable for the estimation of Michaelis constants because of the effects of the EGTA used to control free Ca^{2+} concentrations (9). The average pCa values at half-maximal activity were approximately 5.8 and 5.2, in the presence and absence of calmodulin, respectively, and no significant differences were noted between CF and control data. The (Na + K)-ATPase activity was taken as the difference in ATPase activities at pCa 8.0 in the presence and absence of ouabain.

The results of the ATPase assays are presented for the three study populations in Figure 3 and Table 2. The control Ca-ATPase activities are comparable to those of other investigators using the imidazole lysis buffer for erythrocyte membrane preparation (13). These values are almost 1000 times higher than the activities reported by Katz and Emery (30) but similar to those reported by Foder *et al.* (18). The intersubject variability is also comparable to other reports (36). The Ca-ATPase activities for an individual control subject studied 8 times over a 4-month period using the same conditions of membrane preparation and ATPase assays were: basal Ca-ATPase activity, 29.5 ± 5.9 nmol/min/mg protein, range = 25–40; and calmodulin-stimulated Ca-ATPase activity, 74.8 ± 11.3 nmol/min/mg protein, range = 60–89. The exact source(s) of the variability is unclear, but would appear to include intrasubject changes with time due to unknown factors.

In comparing the CF pancreatic insufficient population with controls, there is a statistically significant decrease in the mean basal Ca-ATPase activity (20%, $p < 0.02$) as there is in the mean calmodulin-stimulated Ca-ATPase activity (23%, $p < 0.001$). However, the mean Ca-ATPase activities for the CF-pancreatic sufficient population are intermediate between the control and pancreatic insufficient patients and are not statistically distinguishable from controls. The mean basal activities for the two CF groups do not differ significantly ($p > 0.1$) while the differences in calmodulin-stimulated activities approach statistical significance ($p < 0.1$). The (Na + K)-ATPase activities of the

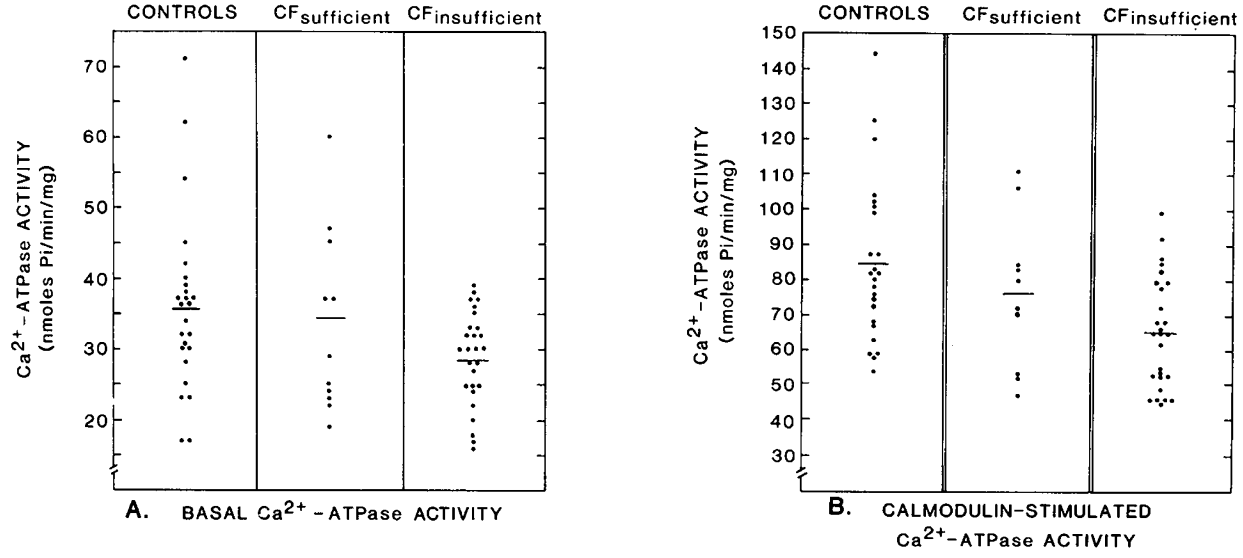

Fig. 3. Maximal Ca-ATPase activities of the three study populations. *A*, basal, Ca-stimulated activities; *B*, Ca-calmodulin-stimulated activities. See the legend to Table 2 for the statistical comparisons.

Table 2. *Comparison of ATPase activities**

	Ca-ATPase		(Na + K)-ATPase
	Basal mean ± SEM, p†	Calmodulin-stimulated mean ± SEM, p†	mean ± SEM, p†
Controls (24)	35.8 ± 2.6	84.3 ± 4.7	13.4 ± 1.1
CF-pancreatic sufficient (10)	35.3 ± 4.2 NS	75.9 ± 6.9 NS	13.3 ± 1.4 NS
CF-pancreatic insufficient (27)	28.9 ± 1.3 <0.02	65.2 ± 3.0 <0.001‡	10.6 ± 0.8 <0.025

* Activities in nmol/min/mg protein; Ca-ATPase activities are maximal values observed as function of pCa (see "Materials and Methods"). Number of subjects is in parentheses; averages of intrasubject values were used where an individual was studied more than once. NS, not significant.

† Unpaired, single-tailed Student's *t* test; compared to controls.

‡ $p < 0.1$ compared to CF-pancreatic sufficient.

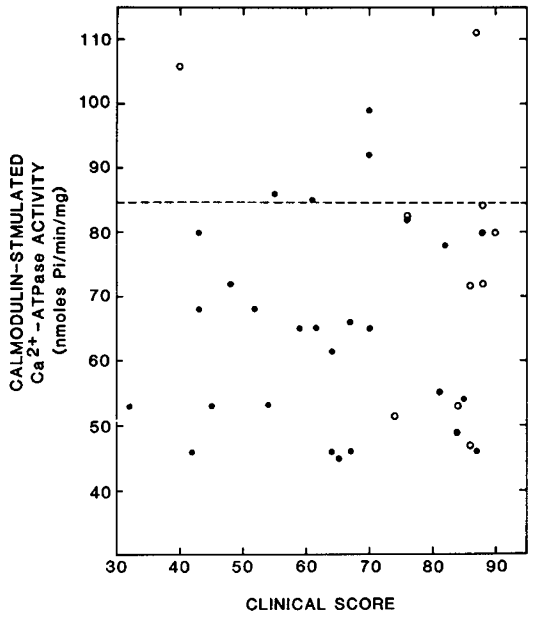

Fig. 4. Maximal calmodulin-stimulated Ca^{2+}-ATPase activities as a function of clinical scores of the cystic fibrosis patients. Pancreatic sufficient patients; O; pancreatic insufficient patients, ●; *dotted line* is the mean activity for the control group.

CF-pancreatic insufficient group also showed a decrease (21%) compared to the control group which was marginally significant ($p < 0.025$); the CF-pancreatic sufficient group did not differ from the control group. No significant correlation was found between any of the ATPase activities and either sex or age within any of the three groups of subjects or for the entire study population.

A difference in general clinical status is one possible source of the different Ca-ATPase activities of the two CF populations since the pancreatic sufficient group had a significantly higher mean clinical score ($p < 0.005$). However, this is not apparent from Figure 4 which compares calmodulin-stimulated Ca-ATPase activities with the clinical scores. Statistical comparison of these two parameters failed to reveal any correlation either within each of the CF groups or for the entire CF study population. Comparison of the Ca-ATPase activities of those patients studied while they were hospitalized for intensive therapy for pulmonary infection with patients studied as outpatients also did not demonstrate any statistical correlations.

DISCUSSION

We have demonstrated a significant decrease in the Ca-ATPase activity, both in the presence and absence of the activator protein calmodulin, in a population of CF patients with pancreatic exocrine insufficiency. In contrast, a subpopulation of CF patients who do not have a history of steatorrhea, do not require pancreatic enzyme replacement, and have normal or high levels of plasma immunoreactive trypsin has average Ca-ATPase activities which are indistinguishable from controls. Foder *et al.* (18), using a method of erythrocyte membrane preparation which should ensure the retention of calmodulin binding to the Ca-ATPase, have found a 15% decrease in maximal Ca-ATPase of CF erythrocyte membranes compared to controls. Our observed decrease of about 25% in the calmodulin-stimulated Ca-ATPase for the pancreatic insufficient patient group may be due to differences in methodology.

While Balfe *et al.* (2) found the activities of ouabain-sensitive ATPase of CF erythrocyte membranes to be decreased by about 30%, subsequent investigators (7, 16, 22, 27, 32, 35) have observed that any decreased (Na + K)-ATPase activities are not significantly different from control activities. Our finding of a marginally significant decrease in the pancreatic insufficient subpopulation probably reflects technical and perhaps patient population differences from these studies.

Previous reports on Ca-ATPase activities in CF erythrocyte membranes other than that of Foder *et al.* (18) did not report attempts to compare the observed activities with any criteria of patient clinical status (1, 7, 16, 24, 27–30, 35). In contrast to our findings of differences based on exocrine pancreatic status, we failed to observe any correlation of Ca-ATPase activities with either general clinical status as documented by a standard four-part clinical scoring system (10, 43) or acute clinical status as judged by the need for hospitalization for intensive pulmonary therapy *versus* comparatively healthy outpatients. This agrees in general with the findings of Foder *et al.* (18) as does our failure to observe any correlation between Ca-ATPase activities and sex or age. However, the high degree of inter- and intrasubject variability together with the rather subjective nature of the clinical criteria may obscure any more subtle, disease-related correlations. Our results together with those of Foder *et al.* (18), who in addition found no Ca-ATPase activity difference between controls and patients with other chronic pulmonary diseases, suggest that advanced lung involvement is not a source of the decreased Ca-ATPase activity.

The large amount of overlap in the activities observed between controls and even the pancreatic insufficient CF patients argues against decreased Ca-ATPase activity being the primary defect in this disorder, although until there is a better understanding of the source of subject variability this cannot be entirely ruled out. Two general explanations for the different mean activities of the two CF subpopulations can be considered. Heterogeneity within CF is well recognized (44) and genetic polymorphism in the Ca-ATPase may be intrinsic to the differences in pancreatic status. Perhaps more likely, the maldigestion and malabsorption accompanying pancreatic deficiency may affect the Ca-ATPase activity through nutritional alterations. Deficient lipid nutrition in these patients has been noted to be reflected in altered fatty acid (25) and phospholipid (39) composition of erythrocyte membranes, decreased circulating and tissue levels of fat-soluble vitamins (especially vitamins E and A) (14, 15, 45) and decreased *in vivo* erythrocyte survival times (14). Studies of both intact erythrocyte membranes (40) and purified, reconstituted Ca-ATPase (18) indicate that the enzyme activity is very sensitive to lipid composition. Similarly, the activity of (Na + K)ATPase is also known to be dependent upon the lipid environment (37, 38). Investigations of these factors in both CF subjects and other patients with pancreatic insufficiency and/or altered lipid nutrition are logical future areas for study.

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