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The nature of the Ca^{2+}-pump defect in the red blood cells of patients with cystic fibrosis

Shmuel Muallem *, Cristina Miner ** and Carol A. Seymour ***

Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG (U.K.) and Department of Medicine, University of Cambridge, Cambridge (U.K.)

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The reduction in $(Ca^{2+} + Mg^{2+})$-ATPase activity in the cystic fibrosis red blood cells can be attributed to a reduction in the number of active Ca^{2+} pumps per red blood cell and an altered interaction of calcium ions with the pump. Despite this, the normal free intracellular $[Ca^{2+}]$ is preserved due to a lower rate of passive calcium entry.

Cystic fibrosis is a well defined clinical entity [1,2]. While secretory abnormalities of some exocrine glands are well documented, the metabolic basis of the disease is unknown [3]. The suggestion that calcium metabolism is altered in cystic fibrosis patients arose from observations of increased Ca^{2+} concentration in the exocrine secretions from patients with cystic fibrosis [4–7]; an increased calcium content of cystic fibrosis skin fibroblasts [8] and a reduced $(Ca^{2+} + Mg^{2+})$-ATPase activity in cystic fibrosis red blood cells [9,10]. However, the nature of the abnormal calcium metabolism in cystic fibrosis is unclear and it is not known whether free intracellular Ca^{2+} levels are affected. This has particular significance since intracellular calcium ions are involved in many physiological functions [11–14].

An ATP-driven Ca^{2+} pump in the plasma membrane has been found in many cells [15] and under physiological conditions plays a major role in controlling the free $[Ca^{2+}]_i$ [16,17]. Thus characterization of the behaviour of the Ca^{2+} pump under pathological conditions may contribute to an understanding of the changed calcium metabolism in these circumstances. The red blood cell provides the simplest system in which calcium transport and $(Ca^{2+} + Mg^{2+})$-ATPase activity can be studied, both in the intact cell and in membrane fragments.

In this paper, we report an analysis of the abnormal behaviour of the calcium pump in the red blood cells of patients with cystic fibrosis by measuring: (a) the number of active calcium pumps per cell as estimated from the level of [^{32}P]phosphoenzyme in the presence of Ca^{2+} and La^{3+} ions and (b) the free $[Ca^{2+}]_i$ and pump-leak turnover by means of an incorporated Ca-chelator BENZ-2 (an aceto-methoxy tetraester) [16]. The patients all had the established clinical features of cystic fibrosis and an abnormal sweat test. Their age range was between 4 months and 18 years old. About half of the patients were taking symptomatic treat-

* Present address: Center for Ulcer Research and Education, Veterans Administration Center, Building 115, Room 217, Los Angeles, CA 90073, U.S.A.
** Present address: Facultad de Medicina, Departamento de Fisiologia y Bioquimica, Valladolid-5, Spain.
*** To whom correspondence should be addressed.

ment at the time of this study, but none were taking antibiotics, prednisolone or diuretic agents.

Table I compares the $(Ca^{2+} + Mg^{2+})$-dependent ATP hydrolysis and the level of $(Ca^{2+} + La^{3+})$-dependent phosphoenzyme in the membrane of red blood cells from control subjects and patients with cystic fibrosis. ATPase activity was measured both in membranes stripped of calmodulin and in similar membranes which had been allowed to rebind calmodulin.

The phosphoenzyme level was measured in the presence of La^{3+} ions, since these ions inhibit specifically the dephosphorylation sequence in the turnover cycle of the pump [18,19]. This effect causes all active pumps to be trapped in the phosphorylated form, so that the number of active calcium pumps per mg red blood cell membrane protein can be estimated. This method has the advantage of measuring the absolute number of active pumps in unpurified membranes irrespective of the normal steady-state level of the phosphoenzyme, the kinetic properties of the pump, and the precise concentrations of pump-ligands such as Ca^{2+} and ATP present during the measurement.

The results in Table I indicate that reduction in $(Ca^{2+} + Mg^{2+})$-ATPase activity in cystic fibrosis red blood cells is due to a reduction in the number of active calcium pumps per mg membrane protein. A similar reduction in $(Ca^{2+} + Mg^{2+})$-ATPase activity of cystic fibrosis membranes is found in the presence and in the absence of calmodulin in these experimental conditions, i.e. saturating concentrations of Ca^{2+} and calmodulin (but see below).

The theoretical possibility that the reduced levels of ATPase activity and ^{32}P incorporated per mg of membrane protein in the cystic fibrosis cells merely reflected the presence of extra protein attached to the membrane was excluded by separate experiments showing that the amount of membrane protein per unit volume of packed cells in cystic fibrosis patients was 0.95 ± 0.15 $(n = 7)$ of the amount present per unit volume of control cells. The results do not indicate whether the 35–38% reduction in the number of active pumps in cystic

TABLE I

RELATION BETWEEN $(Ca^{2+} + Mg^{2+})$-ATPase ACTIVITY AND Ca^{2+}-DEPENDENT PHOSPHOENZYME LEVEL FOR CYSTIC FIBROSIS AND CONTROL RED BLOOD CELLS

Preparation of red cell membranes, measurements of $(Ca^{2+} + Mg^{2+})$-ATPase activity and of the level of phosphoenzyme were as described previously [19]. For the ATPase measurements, membranes were incubated in a medium containing (mM): NaCl, 50; KCl, 50; Hepes-Na (pH 7.6), 10; ouabain, 0.1; $MgCl_2$, 1.0; ATP, 1.0; and either EGTA 0.2 or $CaCl_2$ 0.05, and without or with addition f purified calmodulin 2.5 μg. The $(Ca^{2+} + Mg^{2+})$-dependent ATPase activity was obtained by subtraction of the amount of ATP hydrolysed in the presence of EGTA and Mg^{2+} from the total ATP hydrolysed in the presence of Ca^{2+} and Mg^{2+}. For estimation of the $(Ca^{2+} + La^{3+})$-dependent phosphoenzyme level, about 1 mg membranes were incubated in 0.5 ml of a medium containing (mM): Hepes-Tris (pH 7.6), 100; KCl, 50; and either $MgCl_2$, 0.15 and EGTA 0.1 or $MgCl_2$ 0.05; $CaCl_2$, 0.05 and $LaCl_3$, 0.1. The phosphorylation reaction was initiated by addition of ATP (containing 10^6 cpm [γ-^{32}P]ATP) to a final concentration of 2 μM. After 4 s of incubation at room temperature, the reaction was terminated by rapid addition of 5 ml of a solution containing 5% (v/v) perchloric acid, ATP 1 mM and potassium phosphate 10 mM. The precipitated membranes were collected, washed and the amount of the incorporated ^{32}P measured as described before [19]. At the end of the experiment the protein concentration in each tube was determined by the method of Lowry et al. [20]. The $(Ca^{2+} + La^{3+})$-dependent phosphoenzyme level was calculated by subtraction of the amount of ^{32}P incorporation in the EGTA-containing medium from that in $(Ca^{2+} + La^{3+})$-containing medium. The number of patients/subjects is shown between parentheses.

Red blood cells	$(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol·(mg protein)$^{-1}$·h^{-1})		$(Ca^{2+} + La^{3+})$-dependent phosphoenzyme (pmol·(mg protein)$^{-1}$)
	without calmodulin	with calmodulin	
Controls	1.52 ± 0.18 (4)	8.48 ± 1.11 (7)	5.98 ± 0.27 (7)
Cystic fibrosis	0.98 ± 0.14 (4)	5.28 ± 0.55 (9)	3.85 ± 0.21 (9)
	$p < 0.01$	$p < 0.001$	$p \ll 0.001$
Relative reduction in cystic fibrosis (%)	36 ± 12	38 ± 10	36 ± 5

fibrosis cells represents a total loss of pumps or the presence of non-functioning pumps.

With the reduction in number of active pumps and an unchanged passive calcium permeability, an increase in $[Ca^{2+}]_i$ would be expected. Fig. 1 shows a typical experiment in which the passive calcium permeability and kinetics of pump-leak turnover near steady-state conditions were measured by loading intact red blood cells with Ca-chelator by the non-disruptive technique of Lew et al. [16]. Knowing the total cell calcium content near steady-state, an estimation of free intracellular $[Ca^{2+}]$ can be made from measurements of the internal chelator concentration and of its in situ calcium binding properties. The passive Ca^{2+} influx can be obtained from the initial slope of the net Ca^{2+} influx curve. The Ca^{2+}-pump mediated Ca^{2+} extrusion rate can be assessed from the net Ca^{2+} efflux curve after addition of excess EGTA over Ca^{2+}. As shown before [16,21] analysis of such a curve shows that the pump-mediated flux (ϕ) dependence on $[Ca^{2+}]_i$ is well described by the equation $\phi = A[Ca^{2+}]_i$, where A has the meaning of ϕ_{max}/K_m^2.

A summary of the data obtained from the experiment of Fig. 1 and two other experiments with

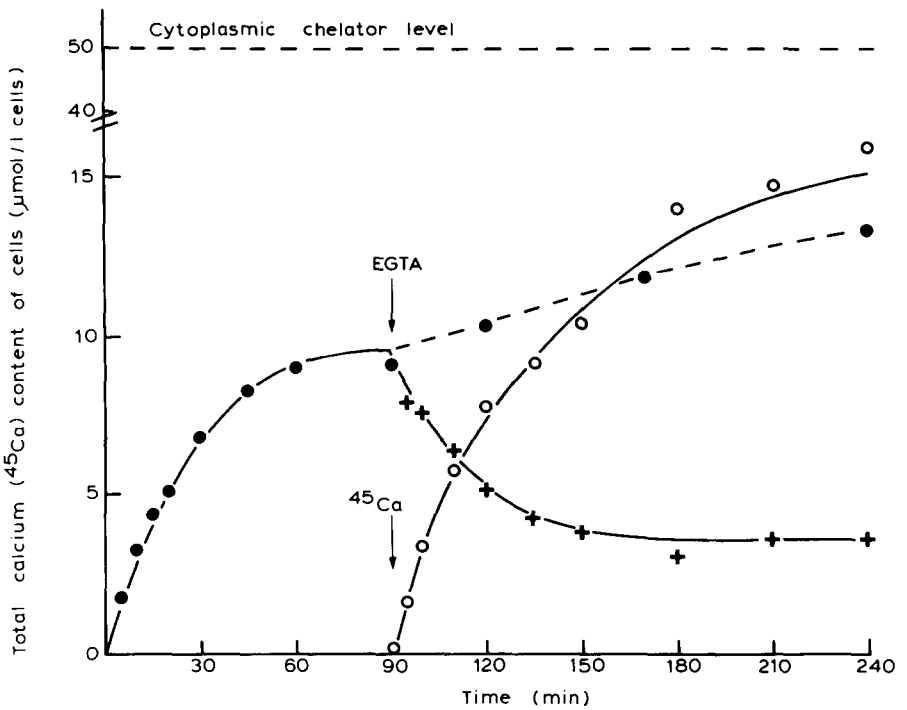

Fig. 1. Time-dependent Ca^{2+} influx into chelator-loaded red blood cells, from patient with cystic fibrosis, suspended in patient's plasma. Non-disruptive loading of red blood cells with Ca^{2+}-chelator agent and the estimation of the intracellular chelator concentration were carried out as described previously [16], with the exception that the ATP depletion step and the electrochemical equilibration with extracellular Ca^{2+} were effected in an incubation medium which also contained 2 mM cysteine (pH 7.4, 37°C). After chelator loading in saline medium, one portion of the cells was resuspended in the original plasma with the addition of 10 mM glucose. The second portion of cells was resuspended in original plasma with 10 mM glucose and ^{45}Ca and time-dependent calcium influx was measured at 37°C with continuous stirring of the sample. 0.2 ml samples were taken into cold saline for measurement of total calcium content of the cells (●) At 90 min, ^{45}Ca was added to cells incubated without tracer (○) for measurement of tracer equilibration fluxes and in parallel 4 mM EGTA was added to one portion of cells incubated with ^{45}Ca (+) for measurement of pump-mediated Ca^{2+} efflux. The EGTA addition was made with a slight excess of NaOH, so as to prevent pH changes after Ca-chelation. A pH of about 7.4 was maintained throughout the period of incubation by titration with HCl. At the end of the incubation period, cells were washed and the calcium content calculated, as in Ref. 16. After 90 min there is an additional non-specific accumulation of Ca^{2+} which is due to prehaemolytic changes of the red blood cells (●— — —●). This is also reflected in the rate of ^{45}Ca distribution measured after 90 min pre-incubation (○).

cystic fibrosis red blood cells is shown in Table II. From these data, intact cystic fibrosis red cells also show a reduction in calcium pumping activity. Furthermore, near the steady-state $[Ca^{2+}]_i$, which is below saturation, the cystic fibrosis red blood cells show a 75% reduction in rate of calcium pumping instead of that expected from the 35–38% reduction in the number of active calcium pumps. This would suggest that in cystic fibrosis red blood cells either K_{Ca} or the activation of the pump by Ca^{2+} or both differ from normal red blood cells. Values for A of $6.8 \cdot 10^{-2}$ and $2.0 \cdot 10^{-4}$ in control and cystic fibrosis red blood cells, respectively, also reflect this. The observed difference in A must be largely due to a higher K_{Ca} in cystic fibrosis cells since the V_{max} of cystic fibrosis (Ca^{2+} + Mg^{2+})-ATPase is 60% that of normal cells (see Table I). Since the K_{Ca} of the Ca^{2+} pump depends on the degree of saturation with calmodulin [24] these results cannot distinguish between an altered affinity for Ca^{2+} by the pump and an abnormal activation by calmodulin. Previous reports claim the same K_{Ca} in cystic fibrosis as in normal cells, but measurements of Ca^{2+} activation of the pump were made at saturating concentrations of calmodulin and only at Ca^{2+} levels far above those in the intact cell, at which all the pumps are bound with calmodulin [22]. Since calmodulin modulation depends on the internal free Ca^{2+} concentration and this is only significant at the physiological Ca^{2+} levels [23,24], our results may also reflect an altered activation pattern of cystic fibrosis red cell Ca^{2+} pump by calmodulin at the $[Ca^{2+}]_i$ levels found in the intact cell.

Despite the demonstrated failure of calcium pumping activity in cystic fibrosis cells, the free intracellular $[Ca^{2+}]$ is in the same range as that for normal red blood cells (Table II) and this has now been described in other cells in cystic fibrosis [25,26]. This results from a simultaneous reduction in passive calcium permeability, similar in magnitude to the reduction in Ca^{2+} pumping activity. The observed reduction in passive calcium permeability seems to be a feature of the plasma membrane itself and not due to any factor present in the plasma of cystic fibrosis patients [27–29]. Thus, plasma from cystic fibrosis patients did not affect the passive calcium permeability of control cells and a similar reduction in passive calcium permeability was found when it was measured with cystic fibrosis red cells suspended in saline (not shown in Fig. 1).

The observation of concomitant changes in passive calcium permeability and the number of calcium pumps supports the interesting suggestion of a link between pump and permeability mechanisms [30]. This, in cystic fibrosis red cells, could be an indication of the way in which this membrane reacts to the defect in Ca^{2+} pump activity so that the reduction in number of active Ca^{2+} pumps is accompanied by a reduction in the passive calcium permeability.

It has been reported that in cystic fibrosis skin fibroblasts the calcium content is increased [8] and

TABLE II

CHARACTERISATION OF THE Ca^{2+} FLUXES IN RED CELLS FROM CYSTIC FIBROSIS PATIENTS AND CONTROL SUBJECTS

The kinetic parameters for control cells were taken from Ref. 16 and those for cystic fibrosis cells from three similar experiments to that shown in Fig. 1 with calculations as explained in the text.

Red blood cells	Steady-state pump leak turnover rate, $\phi(\mu mol \cdot l^{-1} \cdot h^{-1})$	A	Tracer equilibration flux ($\mu mol \cdot l^{-1} \cdot h^{-1}$)	Initial Ca^{2+} influx ($\mu mol \cdot l^{-1} \cdot h^{-1}$)	$[Ca^{2+}]$ at steady-state (nM)
Control (Ref. 16)	45.0	$6.8 \cdot 10^{-2}$	48.0	56.0	26.0
Cystic fibrosis	10.0	$2.0 \cdot 10^{-4}$	19.0	19.0	24.0
Cystic fibrosis	10.6	–	20.8	22.0	22.0
Cystic fibrosis	13.0	–	15.5	17.2	–
Mean ± S.D.	11.2 ± 1.6		18.4 ± 2.7	19.4 ± 2.4	

fibroblast mitochondria accumulate and retain three times as much calcium as normal [31]. However, these findings do not reflect the status of the free $[Ca^{2+}]_i$ level, which at steady state is determined by the plasma membrane Ca^{2+} pump and leak mechanisms. A normal cytosolic free Ca^{2+} in cystic fibrosis fibroblasts [31], but an increased total Ca^{2+} [8], most of it sequestered in the mitochondria [31], suggest an additional protective mechanism present in eukaryotic cells.

Our results seem to indicate that failure of Ca^{2+} pumping in cystic fibrosis red cells does not cause the expected increased $[Ca^{2+}]_i$ levels. If it is justifiable to extrapolate these findings to other cells involved in cystic fibrosis, together with recent measurements of $[Ca^{2+}]_i$ in lymphocytes in cystic fibrosis [25,26] then changes in free $[Ca^{2+}]_i$ are not likely to account for the expression of the disease process in cystic fibrosis.

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