RELATIVE DEFICIENCY OF Ca^{2+}-DEPENDENT ADENOSINE TRIPHOSPHATASE ACTIVITY OF RED CELL MEMBRANES IN HEREDITARY SPHEROCYTOSIS*

Stephen A. Feig[a,b,c] and Guido Guidotti[b]

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SUMMARY: The Ca^{2+}-dependent adenosine triphosphatase activity associated with the plasma membrane of normal human erythrocytes is similar to that of erythrocytes from patients with hereditary spherocytosis. When spherocytic ghosts are compared to age-matched controls, however, they show a significantly decreased Ca^{2+}-dependent adenosine triphosphatase activity. The role of the relative deficiency of Ca^{2+}-dependent adenosine triphosphatase in spherocytic ghosts is discussed in the light of the effects of intracellular [Ca^{2+}] on the deformability and the rigidity of the cell membrane. This enzyme may be involved in the molecular mechanism of hereditary spherocytosis.

INTRODUCTION: Although hereditary spherocytosis is one of the most common congenital hemolytic anemias, the molecular mechanism of this disease has not been defined. Several lines of evidence suggest that the basic defect occurs in the proteins of the red cell membrane (1-4).

There are two general ways to interpret these results: hereditary spherocytosis may be associated with an alteration in a fibrous protein in the membrane which regulates the fluidity and deformability of the membrane, or there might be a change in a

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a) The Gwynne Hazen Cherry Memorial Laboratory, the Department of Pediatrics, UCLA School of Medicine, Los Angeles, California 90024

b) and the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

c) Reprint address: Department of Pediatrics, UCLA School of Medicine, Los Angeles, California 90024.

membrane enzyme which regulates the intracellular level of some substance, like Ca^{2+}, which in turn affects the state of the membrane.

Since there appears to be a relationship between the levels of intracellular ATP, Ca^{2+}, and Mg^{2+} and the deformability of the red cell (5), it has been suggested that an actomyosin-like system is responsible for the ATP-dependent shape changes in cells (6,7). In fact there are several experimental findings which support this suggestion (8-14) and there do seem to be fibrous proteins associated with the red cell membrane (15-17). It can be supposed that there might be an alteration in these actomyosin-like or fibrous proteins in hereditary spherocytosis and this has been suggested by Jacob, Amsden, and White (18). On the other hand, the deformability of red cell membranes seems to be related to the intracellular [Ca^{2+}] (19), which is regulated by an ATP-dependent Ca^{2+} transport mechanism in red cell membranes (20-22). The Ca^{2+} concentration could mediate the degree of deformability of the cell membranes by interacting with either the lipid or the protein components of the membrane. Indeed, red cell Ca^{2+} is virtually completely membrane-associated (23). Furthermore, the increase in membrane permeability and decrease in membrane deformability observed in metabolically depleted erythrocytes is reversed by the chelation of red cell Ca^{2+} with EDTA (5). Therefore, hereditary spherocytosis might be associated with an alteration of the Ca^{2+}-dependent adenosine triphosphatase involved in Ca^{2+} transport.

Since Ca^{2+}-dependent adenosine triphosphatase activities could be related either through the membrane fibrous proteins or the Ca^{2+}pump to the state of the cell membrane, and since the membrane is altered in hereditary spherocytosis, we have studied the Ca^{2+}-adenosine triphosphatase activity of the membrane of patients with hereditary spherocytosis. The data indicate that ghosts made from spherocytic red cells are relatively deficient in Ca^{2+}-adenosine triphosphatase activity compared to appropriate controls, matched for cell age.

MATERIALS AND METHODS: Ghosts were prepared by the method of Nakao,

Kurashina and Nakao (24), from heparinized blood specimens and analyzed without storage. Stromal protein content was assayed by the method of Lowry et al (25).

Red cells were separated according to cell age by centrifugation (26) and the mean cell age of the top and bottom 10% of the cells was estimated by the erythrocyte glutamic oxaloacetic transaminase (EGOT) activity (27).

Ca^{2+}-and Mg^{2+}-adenosine triphosphatase (ATPase) activities of

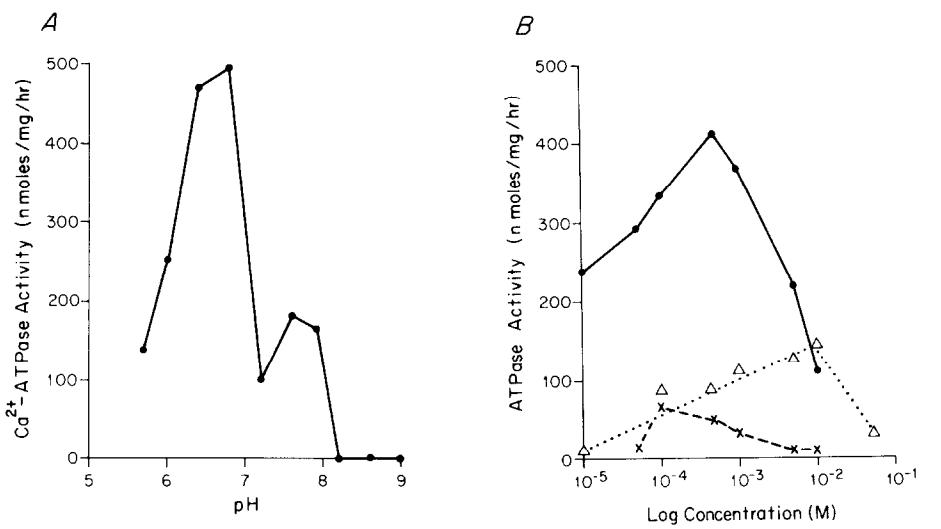

Fig. 1. A. The effect of pH on Ca^{2+}-ATPase. The assay was performed as described in the text except that the Tris-Maleate Buffer was adjusted to provide the desired pH. The reaction was done in a final volume of 0.5 ml at 37°. The reaction was initiated by the addition of Tris-ATP and it was stopped at 30 minutes by the addition of 0.5 ml of cold 10% TCA. The concentration of Pi in the supernatant was analyzed by the method of Ames and Dubin (28). Addition of 10^{-4} M ouabain did not reduce the observed ATP activity, indicating that no $(Na^{+} + K^{+})$-ATPase activity was present. Removal of white blood cells (29) or addition of white blood cells (30) did not change the level of ATPase activity. B. The effect of divalent cation concentration on ATPase activity. x-------x, Ca^{2+} in the absence of Mg^{2+}; Δ ..... Δ, Mg^{2+} in the absence of Ca^{2+}; ●——● , Ca^{2+} in the presence of 0.001 M Mg^{2+}.

ghosts were measured in a 0.05 M Tris maleate buffer with 0.001 M EGTA at pH 6.8. This pH was shown to provide maximum Ca^{2+}-ATPase activity (Fig. 1A). Ca^{2+}-ATPase was defined as the Pi liberated from ATP in the presence of Ca^{2+} and Mg^{2+}, less that liberated in the presence of Mg^{2+} alone. The final concentrations of Mg^{2+} (0.01M) and Ca^{2+} (0.001M) were chosen to maximize the reaction rate (Fig. 1B). The final concentration of free Ca^{2+} was calculated from the binding constant of EGTA, 4.4×10^5 M^{-1} (31). For ATP the maximum velocity was achieved at 0.002M, confirming the data of Rosenthal and his associated (13).

Blood samples were obtained from patients at the Pediatric Hematology Clinic of the Children's Hospital Medical Center in Boston, and the UCLA Center for the Health Sciences. Twenty-two patients from 12 families with hereditary spherocytosis were studied. All splenectomized patients had a normal hemoglobin, packed cell volume, and reticulocyte count with persistent spherocytosis, while presplenectomy patients with spherocytosis had reticulocyte counts which ranged from 10 to 30%.

Control specimens were obtained from hematologically normal hospital and laboratory personnel. High reticulocyte controls were obtained from patients with hemolytic conditions other than hereditary spherocytosis. The reticulocyte counts of these samples ranged from 10 to 50%. Splenectomized controls included patients who had undergone splenectomy for non-hemolytic indications (ten of these fourteen patients had Hodgkin's Disease but were not receiving chemotherapy at the time of testing).

RESULTS AND DISCUSSION: Table I shows the results of the enzymatic assays done on the membranes of normal and spherocytic cells. While the Mg^{2+}-ATPase activity of spherocytic ghosts (from splenectomized and non-splenectomized patients) is higher than that of normal ghosts (from splenectomized and non-splenectomized people) the Ca^{2+}-ATPase activity is similar in the two types of cells.

This result could depend on the fact that the spherocytic cells even after splenectomy, represent a younger population of cells than do those from normal people (32, 33) and that while the Mg^{2+}-

TABLE I

Enzyme Activities of Erythrocytes

	Mg^{2+}-ATPase*	Ca^{2+}-ATPase	EGOT#
Normal:			
Normal	220±81 (83)	616±272 (83)	0.56±0.18 (28)
High reticulocyte	369±155 (12)	897±383 (12)	1.45±0.43 (4)
Splenectomized	210±83 (14)	521±237 (14)	-
Hereditary Spherocytosis:			
Pre-splenectomy	322±97 (9)	615±262 (9)	1.38±0.42 (6)
Post-splenectomy	306±95 (17)	556±243 (17)	0.76±0.22 (13)

* Activity expressed as nanomoles Pi/mg ghost protein/hr. ± S.D.
\# Activity expressed as units/10^{10}RBC's/min. ± S.D.
(n) = number

ATPase is age labile the Ca^{2+}-ATPase is not affected by the age of the cell.

In fact, the EGOT activities, an independent measure of mean cell age, indicate that spherocytic cells (from non-splenectomized patients) are generally comparable to high-reticulocyte controls, and that post-splenectomy spherocytic cells are somewhat younger than normal controls, in spite of similar reticulocyte counts. This provides further confirmation of a moderate reduction of mean red cell survival after splenectomy in hereditary spherocytosis (32, 33). However, the Ca^{2+}-ATPase activities of both normal and post-splenectomy spherocytic cells are age labile, since these enzymatic activities are higher in young cells than they are in old cells

TABLE II

Age Lability of Erythrocyte Enzymes[a)]

	EGOT#	Ca^{2+}-ATPase*
Normal (n = 8)		
Top	0.83±0.25	607±231
Bottom	0.47±0.19	480±150
Ratio§	0.55±0.13	0.65±0.17
Hereditary Spherocytosis (n = 8)		
Top	1.02±0.27	672±182
Bottom	0.72±0.21	471±118
Ratio§	0.70±0.09	0.67±0.22

* Activity expressed as nanomoles Pi/mg ghost protein/hr. ±S.D.
\# Activity expressed as units/10^{10}RBC's/min. ±S.D.
§ Expressed as mean ±S.D. of the ratio activity bottom layer/ activity top layer for each sample.
a) Red cells were separated into age groups by ultracentrifugation (26). The top and bottom 10% of cells were harvested to represent the young and old cell populations. The cells were then washed and lysed as described.

separated by differential centrifugation (Table II). This result is in agreement with the observation (Table I) that both Mg^{2+}-and Ca^{2+}-ATPase activities are high, with respect to normal controls, in the cells from the high reticulocyte controls and that both enzymes are affected by the age of the cells.

These data suggest that both the Mg^{2+}-and Ca^{2+}-ATPases are age labile enzymes of the red cell membrane. Previous results by Nakao et al (14) indicated that a ouabain-insensitive ATPase requiring Mg^{2+} but not Ca^{2+} is deficient in spherocytic ghosts. These data were not confirmed in a subsequent study by Wiley (34)

and are not borne out by the present study. In contrast, the Mg^{2+}-ATPase activity appears to be increased in spherocytic membranes commensurate with the relative youth of that cell population. Although the Ca^{2+}-ATPase is similarly age-labile, the specific activity of that enzyme is decreased in spherocytic membranes. Since the age-related decay of that enzyme is normal in spherocytes, this would suggest that the enzyme is deficient at the time of its release from the marrow and not subject to excessively rapid decay.

Admittedly, it would be most unusual for an enzyme defect to be responsible for the pathogenesis of a syndrome inherited as an autosomal dominant. This clinical syndrome is, however, quite innocuous and might not be subject to the generalizations which apply to the more severe enzymopathies.

Whether a relative deficiency of Ca^{2+}-ATPase is a primary defect or simply reflects a physiologic abnormality is uncertain. The current data do not clarify this problem, but they do point strongly to a relationship between the intracellular [Ca^{2+}], the calcium-pump (Ca^{2+}-ATPase), the fibrous membrane protein system, and the state (deformability, rigidity, permeability) of the membrane.

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