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The calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase in hemoglobin S erythrocyte membranes: effects of sickling and oxidative agents

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A decrease in the reactivity of erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase to calmodulin stimulation has been observed in aging red cells and in various types of hemolytic anemias, particularly in sickle red cell membranes. Unlike the aging process, the defect in the (Ca^{2+} + Mg^{2+})-ATPase from SS red blood cells is not secondary to a decrease in calmodulin activity and is already present in the least dense SS red blood cells separated on a discontinuous density gradient. Deoxygenated AS red cells were forced to sickle by lowering the pH, raising the osmolarity of the buffer (sickling pulse). Under these conditions an inhibition of the calmodulin-stimulated enzyme was observed only if several cycles of oxygenation/deoxygenation were applied. No alteration of the enzyme could be detected after submitting AS red blood cells to other conditions or in AA red blood cells submitted to the same treatments. This suggests that oxidative processes are involved in the alterations of the (Ca^{2+} + Mg^{2+})-ATPase activity. Treatment of membranes from AA erythrocytes by thiol group reagents and malondialdehyde, a by-product of auto-oxidation of membrane unsaturated lipids and a cross-linking agent of cytoskeletal proteins, led to a partial inhibition of the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase. We postulate that the hyperproduction of free radicals described in the SS red blood cells and involved in the destabilization of the membrane may be also responsible for the (Ca^{2+} + Mg^{2+})-ATPase failure.

Introduction

A decrease in the activity of the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase from red blood cell membranes has been described during cell senescence and in different cases of hemolytic anemias such as sickle cell disease [1–4].

The Ca^{2+} pump associated with (Ca^{2+} + Mg^{2+})-ATPase activity [5] is the sole active mechanism responsible for the maintenance of the very low intracellular Ca^{2+} concentration (10^{-7} M) in the red blood cell. The increase in the Ca^{2+} content observed in these pathophysiological conditions [6,7] has been related to the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase. Several recent articles have outlined the deleterious effects resulting from the increase in cytosolic [Ca^{2+}] on the erythrocyte membrane and on the survival of the cell in the circulation [8,9]. The inhibition of the (Ca^{2+} +

Abbreviations: AA, AS, SS red blood cells, respectively red blood cells from normal donors, sickle cell trait individuals, and patients homozygous for HbS; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Mg^{2+})-ATPase activity upon aging has been ascribed by Ekholm et al. [10] to a decreased activity of cytosolic calmodulin. In SS red blood cells, calmodulin activity is normal [3,11] and the alteration of the enzyme remains unexplained [2,3,11] and even controversial [12].

In the last few years, various alterations of SS erythrocyte membranes have been demonstrated [13,14]. Oxidative phenomena seem to play a major role in membrane damage occurring in this disease [15]. These oxidative reactions involve not only membrane lipids but also cytoskeletal proteins [15,16]. Oxidation of SH groups of proteins by thiol-reagents results in the inhibition of the plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase from red blood cells and other tissues [17,18]. Therefore the enzyme may also be a target for oxidative radicals overproduced in sickle cell disease [15].

In this study we made an attempt to elucidate the mechanisms leading to the partial inhibition of the ($Ca^{2+} + Mg^{2+}$)-ATPase of SS erythrocytes. We demonstrate that these mechanisms are different from those observed in the aging process. Our results indicate that the defect in the ($Ca^{2+} + Mg^{2+}$)-ATPase activity depends on the sickling process. Attention was paid to the possible oxidation of the protein in the SS erythrocytes and we demonstrate that malondialdehyde, an end-product of lipid peroxidation and a cross-linking agent, is able to induce inhibition of the ($Ca^{2+} + Mg^{2+}$)-ATPase in normal red cell membranes. This is relevant to sickle cell disease, as malondialdehyde is produced in excess in these cells [19].

Materials and Methods

All chemicals used in this study were of analytical grade: Hepes, histidine, imidazole, bovine serum albumin and ouabain were from Sigma (St. Louis, MO, U.S.A.), calmodulin (hog brain), trifluoperazine and vanadium-free ATP were from Boehringer-Mannheim (F.R.G.), and Percoll was from Pharmacia (Sweden).

Blood from healthy non-smoking donors (AA) and heterozygous AS individuals was collected on heparin. SS blood samples were obtained from homozygous patients undergoing blood exchange-transfusion prior to surgery. Homozygous patients were at a distance from crises and had not been transfused for several months. Anemia was moderate (mean Hb concentration = 8.2 ± 0.4 g/dl).

Membrane preparation was achieved within 24 h after blood withdrawal. Blood was washed three times in a cold 10 mM Hepes buffer containing 150 mM KCl, 11 mM glucose, pH 7.40. The buffy coat was removed by aspiration after each centrifugation ($2500 \times g$, 10 min). In one series of experiments the membranes were prepared from the whole population of red blood cells. In a second series the membranes were prepared from the 10% least dense and 10% densest fractions of erythrocytes. Prior to the fractionation a density-profile of the whole population was obtained by the phthalate ester density gradient method [20]. The fractions were then isolated on a discontinuous Percoll-albumin density gradient according to the method used in this laboratory [21].

Sickling pulse

AA and AS red blood cells were treated according to the method described by Bookchin and Lew [22] with some modifications. Packed red blood cells were suspended in a 9 mM Hepes buffer containing 135 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 0.45 mM Tris-HCl, 0.045 mM EGTA, 1% bovine serum albumin, 10 mM glucose, pH 7.8, to obtain a packed cell volume of 10%. When necessary the suspension of red blood cells was deoxygenated by equilibrating for 1 h under pure nitrogen and was then kept deoxygenated or submitted to cycles of oxygenation/deoxygenation every 30 min. Sickling was induced by the rapid injection of a hyperosmotic and acidic solution that brought the osmotic pressure up to 400 mosM and the pH down to 7.1. Control samples were run in oxygenated conditions in isosmotic and hyperosmotic media. Incubation at 37°C was carried out for up to 8 h, after which the sample was withdrawn anaerobically and centrifuged for 10 min at $2500 \times g$. Supernatants were kept for the determination of the hemoglobin content in order to check for the presence of hemolysis. The packed red cells were then hemolyzed for membrane preparation. A scheme of the different conditions applied to the red blood cells is represented in Fig. 1.

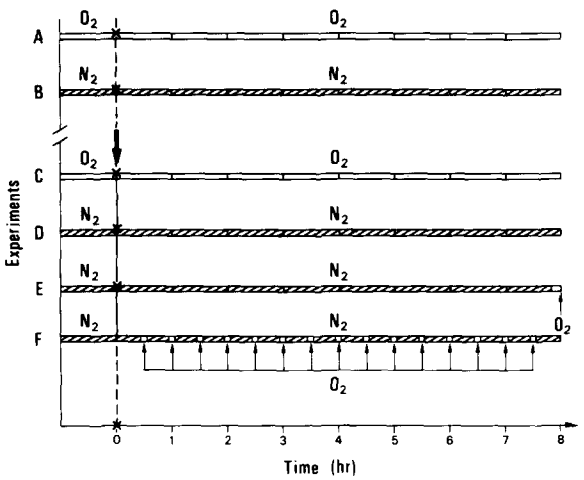

Fig. 1. Scheme of the various experimental conditions applied to AA and AS red blood cells in the sickling pulse experiments. 1 h equilibrium of the red blood cells suspensions with either oxygen or nitrogen was applied before the experiment. Heavy arrow represents the sickling pulse, which consisted of the rapid injection of a hyperosmolar, acidic buffer that brought up the osmolarity to 400 mosM and lowered the pH value to 7.1. Conditions before the sickling pulse are given in the text. Light arrows indicate short periods of oxygenation. Capital letters refer to various experimental conditions.

Preparation of red cell membranes

Erythrocyte membranes were prepared by hemolysis using a 310 mosM imidazole buffer according to Farrance and Vincenzi [23]. The pellet was washed twice in a 20 mM imidazole buffer, pH 7.4, and once in a 40 mM histidine-imidazole buffer, pH 7.1. The membranes were kept frozen in the latter buffer at −80°C. Each membrane preparation was submitted to saponin treatment (1 mg saponin/mg membrane protein) for 20 min at room temperature prior to the assay of the ATPase activity in order to have free access to all $(Ca^{2+} + Mg^{2+})$-ATPase copies.

Measurement of the $(Ca^{2+} + Mg^{2+})$-ATPase activity

The amount of inorganic phosphate (P_i) produced by the hydrolysis of ATP was measured using the malachite green dye method according to Kallner [24]. The activity of the enzyme was expressed as nmol P_i produced per mg protein per min. The reaction assay was performed in the following basic medium: 18 mM histidine-imidazole buffer, 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, 0.1 mM ouabain, 0.1 mM EGTA, pH 7.1 at 37°C for 30 min. Trifluoperazine (10^{-7} M) was added to suppress any remaining calmodulin activity. The reaction was started by adding ATP (final concentration 2 mM). In these basal conditions the amount of P_i produced upon addition of ATP represents the Mg^{2+}-dependent ATPase activity. Calcium-stimulated ATPase activity was measured after adding 20 µl/ml $CaCl_2$ (0.2 mM) to the reaction medium. The calmodulin-stimulated ATPase activity was obtained in the presence of 10^{-8} M hog brain calmodulin and Ca^{2+} without trifluoperazine. The concentration of the different substrates were adjusted to obtain a maximal activation of the enzyme. Total membrane protein was measured according to Bradford [25]. Membrane-bound hemoglobin was determined spectrophotometrically as cyanmethemoglobin at 420 nm. The hemoglobin-free membrane protein was calculated by difference. The concentration of hemoglobin-free membrane protein used in the assay was adjusted to 0.03 mg/ml (final concentration).

Treatment of AA erythrocyte membranes by malondialdehyde and thiol-group reagents

A 4 mM malondialdehyde bis(dimethylacetal) solution was made up in 0.1 M HCl. The hydrolysis was carried out for 1 h at room temperature. The solution was then neutralized by adding Tris base. The concentration of free malondialdehyde was estimated spectrophotometrically at 267 nm ($\epsilon = 34000\ M^{-1} \cdot cm^{-1}$) and adjusted to the concentration under study. Membranes were incubated in the presence of malondialdehyde ranging from 0.2 to 2 mM at 37°C and for 1, 2 or 3 h prior to the assay. Exposure of AA erythrocyte membranes to SH-group reagents, diamide and *N*-ethylmaleimide (0.1 mM), was also carried out for 30 min at 37°C. In parallel experiments, protection against these oxidative effects was attempted by adding 10 mM dithiothreitol (final concentration).

Statistical analysis of the results was done according to Student's *t* test and significance of differences between mean values was estimated at the 5% level.

Results

Comparison of (Ca^{2+} + Mg^{2+})-ATPase activities from AA, AS and SS erythrocyte membranes

Table I shows the different (Ca^{2+} + Mg^{2+})-ATPase activities measured in erythrocyte membranes prepared from blood of normal (AA), heterozygous (AS) and homozygous (SS) adult individuals. For the three types of membranes the Mg^{2+}-dependent ATPase activities were very low and in the same range. Addition of Ca^{2+} enhanced this basal activity to similar levels in all AA, AS and SS RBC membranes.

Exogenous calmodulin (10^{-8} M) enhanced the (Ca^{2+} + Mg^{2+})-ATPase activity in AA and AS membranes to the same extent. The response to calmodulin stimulation of SS membrane (Ca^{2+} + Mg^{2+})-ATPase was present but accounted for only 50% of that observed in the other types of membranes. The activity of the (Ca^{2+} + Mg^{2+})-ATPase in SS membranes was also measured with varying concentrations of calcium, calmodulin and ATP. The results (not shown) revealed no difference in the affinity of the enzyme for its substrate and activators compared to the normal red cell enzyme. In optimal conditions a significant decrease in V_{max} (−30%) was observed, which confirms similar data reported by others [26].

(Ca^{2+} + Mg^{2+})-ATPase activities were measured in membranes prepared from AA and SS red cells separated on a discontinuous Percoll-albumin density gradient (Table II). Optical microscope studies showed an enrichment in irreversibly sickle cells from about 7% in the top to 60–70% in the bottom fractions (500 counted cells). Table II displays the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activities obtained in the top and bottom fractions of each group of cells. No difference related to density could be detected in AA or in SS membranes. Inhibition (−50%) of the calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase in SS membranes could be seen and was identical in the two fractions. These results indicate that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase present in the least dense SS cells precedes the irreversibility of the cell deformation. AS red blood cells, which rarely undergo sickling in normal physiological conditions, exhibit normal (Ca^{2+} + Mg^{2+})-ATPase activities. The inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity observed should therefore be related to sickling secondary to polymerization of hemoglobin S.

Calmodulin-stimulated (Ca^{2+} + Mg^{2+})-ATPase activities from AA and AS red blood cells submitted to a 'sickling pulse'

AA and AS red blood cells were fully deoxygenated and brought to hyperosmolarity and dehydration (sickling pulse). Only when those two conditions were associated did AS cells sickle. Different conditions of oxygenation or of deoxygenation and hyperosmolarity of the incubating buffer have been applied to the cells (Fig. 1). Only cycles of oxygenation and deoxygenation in a hyperosmolar buffer resulted in an alteration of the enzyme stimulation by calmodulin (−23%) (F). A slight decrease was detected when the cells were exposed to pure oxygen after a prolonged incubation (8 h) under pure nitrogen (−15%) (E). Normal red cells were not affected by these procedures (Fig. 2).

TABLE I

Activities of the (Ca^{2+} + Mg^{2+})-ATPase of membrane obtained from AA (red blood cell from normal donors), AS (red blood cell from sickle cell trait individuals) and SS (red blood cell from sickle cell patients) were measured as described in Methods. Values are the means ± S.E. of 4–6 different membrane preparations

	(Ca^{2+} + Mg^{2+})-ATPase activities (nmol P_i/mg membrane protein per min)		
	Mg^{2+}-dependent	Ca^{2+}-stimulated	Calmodulin-stimulated
AA (n = 6)	3.3 ± 1.2	29.7 ± 5.0	129.1 ± 12.6 [a]
AS (n = 5)	4.5 ± 1.3	38.3 ± 5.9	138.9 ± 11.2
SS (n = 4)	8.5 ± 2.4	24.5 ± 4.0	77.1 ± 4.5 [a]

[a] Comparison between calmodulin-stimulated activities from AA and SS red blood cell membranes $P < 0.01$.

TABLE II

CALMODULIN-STIMULATED ($Ca^{2+} + Mg^{2+}$)-ATPase OF MEMBRANES PREPARED FROM THE TOP AND BOTTOM FRACTIONS OF AA AND SS RED BLOOD CELLS

Least dense and densest fractions of red blood cells were separated on a discontinuous Percoll-albumin gradient. ($Ca^{2+} + Mg^{2+}$)-ATPase activities are expressed as nmoles P_i/mg membrane protein per min. Results are means ± 1 S.D.

	Calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activities ([CaM] = 10^{-8} M)	
	AA membranes ($n = 3$)	SS membranes ($n = 2$)
Least dense fraction (10%)	97 ±11.7	36.3 ± 11.2
Densest fraction (10%)	86.8 ± 0.8	33 ±10.5

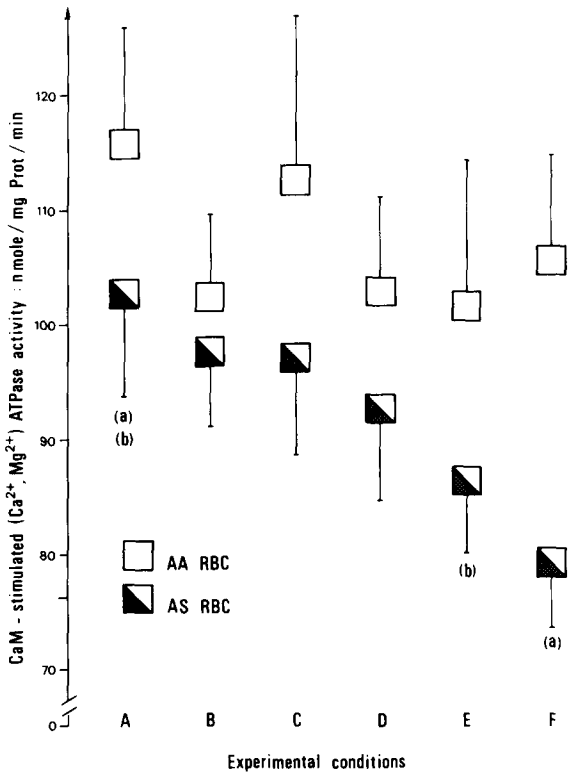

Fig. 2. Decreased activity of the calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase from AS red blood cells submitted to cycles of oxygenation/deoxygenation after a sickling pulse. Experimental conditions are described in Fig. 1. (a) Comparison of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activities resulting from AS erythrocytes submitted to the sickling pulse and the cycles of oxygenation/deoxygenation (F) with their own control (A). $P < 0.01$. (b) Comparison of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activities resulting from AS erythrocytes submitted to the sickling pulse and deoxygenation for 8 h followed by reoxygenation (E) with their own control (A). $P < 0.02$. Values are means ± S.E. of six (AS erythrocytes) and seven (AA erythrocytes) experiments.

Inhibition of the ($Ca^{2+} + Mg^{2+}$)-ATPase from normal red blood cells by thiol-reagents and malondialdehyde

Since malondialdehyde is an end-product of the oxidation of unsaturated membrane lipids and a cross-linking agent of the cytoskeletal proteins we studied its effects on the ($Ca^{2+} + Mg^{2+}$)-ATPase activity of normal red blood cell membranes. In the presence of MDA the enzyme activity is inhibited (Table III). The inhibition depends on the concentration and the duration of malondi-

TABLE III

EFFECT OF MALONDIALDEHYDE ON CALMODULIN-STIMULATED ($Ca^{2+} + Mg^{2+}$)-ATPase ACTIVITY FROM AA RED BLOOD CELLS

Percentages of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activity remaining after treatment of AA erythrocyte membranes with malondialdehyde (MDA). For each incubation time the reference is the enzyme activity of the untreated AA erythrocyte membrane (set to 100%). Results are means ± S.E. for six different experiments.

Duration	Percentage of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activity				
[MDA]:	0	0.2 mM	0.4 mM	1 mM	2 mM
60 min	100	91.0 ± 2.9	83.8 ± 5.1	81.5 ± 3.4	66.6 ± 6.3
120 min	100	92.8 ± 2.4	84.5 ± 3.3	71.2 ± 3.1	57.4 ± 6.7
180 min	100	93.8 ± 3.9	84.5 ± 4.2	61.6 ± 6.4	38.0 ± 3.9

TABLE IV

COMPARATIVE EFFECTS OF DIAMIDE, *N*-ETHYLMALEIMIDE AND MALONDIALDEHYDE ON CALMODULIN-STIMULATED ($Ca^{2+} + Mg^{2+}$)-ATPase FROM ONE AA RED BLOOD CELLS MEMBRANE PREPARATION

Percentages of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activity from AA erythrocyte membrane treated: (1) with diamide (0.1 mM), *N*-ethylmaleimide (NEM) (0.1 mM) for 30 min at 37°C and malondialdehyde (MDA) (2 mM) for 3 h at 37°C; (2) with the previous protein oxidants + dithiothreitol (DTT) (10 mM). 100% represents the CaM-stimulated enzyme activity after incubation of untreated AA membrane for respectively 30 min (diamide, *N*-ethylmaleimide) or 3 h (malondialdehyde).

	Percentage of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activity			
	control	diamide, 0.1 mM, 30 min	NEM, 0.1 mM, 30 min	MDA, 2 mM, 3 h
	100	10.5	53.4	38.6
+ DDT (10 mM)	101.5	84.7	63.1	41.2

aldehyde application. Different thiol-reagents and cross-linking agents (diamide, *N*-ethylmaleimide) have been shown to inhibit the ($Ca^{2+} + Mg^{2+}$)-ATPase [17,18] which contains 7 SH-groups. The effect of malondialdehyde on ($Ca^{2+} + Mg^{2+}$)-ATPase was compared to that of *N*-ethylmaleimide and diamide (Table IV). Addition of a thiol-group protector such as dithiothreitol prevented the inhibition of the enzyme by diamide but not that by *N*-ethylmaleimide or malondialdehyde as expected. We also applied dithiothreitol treatment to SS membranes. This procedure did not reverse to normal the calmodulin-stimulated enzyme activity: 70.6 ± 4.4 nmol/mg protein per min in control SS red blood cells membranes versus 71.9 ± 2.4 nmol/mg protein per min in dithiothreitol-treated ones ($n = 4$).

Discussion

In agreement with previous reports [2,3,11], the present results reveal a 50% inhibition of calmodulin-stimulated ($Ca^{2+} + Mg^{2+}$)-ATPase activity in membrane from SS red blood cells. The inhibition of the enzyme was not related to the cell density or to the irreversibility of the membrane damage, since it was observed in the least dense and still deformable cells. Using exogenous calmodulin our results rule out the possibility of a defect in the calmodulin activity from SS red blood cells like that observed in the senescent cells [10]. In contrast with our results, increased ($Ca^{2+} + Mg^{2+}$)-ATPase activities in SS erythrocytes have been reported by Luthra and Sears [12]. These authors expressed enzyme activities as μmol P_i per g hemoglobin per 2 h instead of nmol P_i per mg membrane protein per min. The former expression may be misleading as the mean corpuscular volume of SS red cells is frequently lower than normal due to associated thalassemia. This could result in a higher protein/hemoglobin ratio in the membrane. Calculating on a hemoglobin basis, our results became similar to those of Luthra and Sears. Traces of hemoglobin always remain in ghost preparation, especially in SS red blood cells. In our hands the presence of membrane-bound hemoglobin was almost undetectable for AA and AS erythrocytes even after a 'sickling pulse' and cycles of oxygenation/deoxygenation; it did not exceed 10% of the total amount of protein in SS erythrocytes membranes. It should be stressed that measurements in the visible spectrum reflect the amount of total heme, not necessarily bound to globin. Our calculations assumed that the heme concentration corresponded to that of hemoglobin. Taking these factors into consideration, the present values for the calmodulin-stimulated Ca^{2+}-ATPase activity should be rather overestimated. Furthermore, we could not demonstrate any relationship between the enzyme defect and the amount of membrane-bound hemoglobin.

Our results are in full agreement with those reported by Niggli et al. [26]. Using isolated ($Ca^{2+} + Mg^{2+}$)-ATPase preparations these authors pro-

vided a definite proof for a decrease of the activity of the enzyme in sickle cell disease. The normal activity of the $(Ca^{2+} + Mg^{2+})$-ATPase from AS erythrocytes suggests that the alterations of the enzyme in SS erythrocytes are related to the sickling process. This hypothesis is supported by the appearance of an inhibition of the calmodulin-stimulated enzyme in AS cells forced to sickle. The part played by the failure of the $(Ca^{2+} + Mg^{2+})$-ATPase in the elevated Ca^{2+} content of the SS red blood cell is still a matter of debate. Bookchin and Lew have used a 'sickling pulse' to study Ca^{2+} transport in AS erythrocytes [22]. At low pH, high ionic strength and after deoxygenation they showed that the sickling process induced a large uptake of calcium and an irreversible inhibition of the Ca^{2+} transport in these cells, whereas Ca^{2+} movements in normal erythrocytes were not altered. Contrasting with our experiments, the alteration of the Ca^{2+} transport appeared as a more rapid event (less than 2.5 h) and did not require cycles of oxygenation/deoxygenation. These authors have proposed recently [27] that the increase in calcium content of sickle red cells may be explained by a sequestration of the cation in endocytic inside-out vesicles. This mechanism prevents any control of total $[Ca^{2+}]$ by the plasma membrane Ca^{2+} pump. Rhoda et al. [28], using Ca^{2+}-selective chelators, did not find an increased cytoplasmic free Ca^{2+} level in oxygenated SS red blood cells. This interpretation is, however, tempered by recent observations by Varecka and Carafoli [29] demonstrating that a partial inhibition of the ATPase by vanadate leads to a large increase in the calcium cellular content in AA red blood cells [29].

Cycles of oxygenation/deoxygenation are necessary to produce an inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase in AS red cells. Ohnishi recently demonstrated [30] that after exposure of SS red cells to repeated cycles of oxygenation/deoxygenation the number of irreversible sickle cells was augmented only in the presence of calcium.

During the sickling event important modifications of the organization of the cell membrane phospholipids have been described [13,31,32], possibly related to an increased production of oxidative radicals [15]. In normal erythrocytes, Haest et al. [33] demonstrated that treatment with diamide led to a dimerization of the spectrin, inducing a destabilization of the membrane and a loss of the phospholipid asymmetry with the appearance of phosphatidylserine in the outer leaflet of the membrane. We therefore postulated that the oxidative mechanisms described in SS red cell membranes [15,19] might be responsible at least in part for the inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase in SS red cells. This is supported by observations showing an inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase in glucose-6-phosphate dehydrogenase deficient red cells, in which an increased production of free radicals has been measured [4]. The oxidative processes may involve thiol groups in the enzyme as indicated by the effect of *N*-ethylmaleimide, diamide and malondialdehyde (Table IV). However, the inhibition of the SS red cells enzyme was not reversed upon addition of the potent reducing agent dithiothreitol. Therefore cross-linking reactions are more likely to be involved in the inhibition of the enzyme. In fact oxidizing agents may be responsible for cross-linking of amino groups in the membrane proteins, as already shown for spectrin, leading to gross structural alterations [16].

Altogether, these various factors may contribute to inhibiting the red cell $(Ca^{2+} + Mg^{2+})$-ATPase in sickle cell disease. Further studies on the isolated enzyme from sickle red cells are necessary to unveil the exact mechanism of this inhibition. Due to the deleterious effects of an increased Ca^{2+} level in red cells, the partial inhibition of the enzyme may be relevant to the pathology of sickle cell disease.

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