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Divalent Cation Dependent ATPase Activities of Red Blood Cell Membranes: Influence of the Oxidation of Membrane Thiol Groups Close to Each Other

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An Mg^{2+}-dependent low ATPase activity can be detected in erythrocyte "white membranes," in addition to that of the well known $(Ca^{2+} + Mg^{2+})$-ATPase. The thiol oxidizing agent diamide affects both activities. The oxidation of neighboring thiols seems to leave the mechanism of the $(Ca^{2+} + Mg^{2+})$-ATPase amplification system evoked by Ca^{2+} largely unaffected. Tne perturbation caused by diamide in the membranes seems to affect primarily a step of the ATP hydrolysis mechanism that is common to both ATPase activities. The effectiveness of diamide seems to be the same when either Ca^{2+} and Mg^{2+}, or Mg^{2+} alone are present during the reagent action. Reduction of disulfide bonds by DTE after diamide treatment restores the $(Ca^{2+} + Mg^{2+})$-ATPase activity but is unable to take the Mg^{2+}-ATPase activity back to the original level.

The hypothesis is discussed that the redox state of one (or more than one) couple of –SH close to each other and possibly connected to the active site, may be an important factor in optimizing the efficiency of Ca action on the $(Ca^{2+} + Mg^{2+})$-ATPase.

Key words: red cell membranes, ATPase, Ca^{2+}, Mg^{2+}, diamide

The ATPase activities of human erythrocyte membranes have been extensively studied by numerous workers. It has been reported by many authors that the divalent cations Ca^{2+} and Mg^{2+} are responsible for the regulation of the outwardly directed CA^{2+} pump and are probably associated with the contractile activity of some proteins described in the red blood cell membrane [1–7]. There is evidence that ATP acts as the direct source of energy for the above-mentioned systems, whose activity may conceivably be

Abbreviations used: DTE, 2,3-dihydroxy-1,4 dithiolbutane; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N-etraacetic acid; EDTA, ethylene diamino-tetraacetic acid; Tris, tris (hydroxy methyl)-aminomethan; DTNB, 2,2'-dinitro-5,5'-dithiobenzoic acid; diamide; diazene dicarboxylic acid N,N-dimethylamide; SDS, sodium dodecyl sulphate.

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reflected by the divalent cation-dependent ATPase activity detectable in isolated red blood cell membranes [8, 9]. A mechanism for a four step reaction sequence implicating Ca^{2+} and Mg^{2+} in the formation and lysis of the phosphorylated intermediate has been proposed by Rega and Garrahan [10] for the $(Ca^{2+}+Mg^{2+})$-ATPase. Moreover, the ATPase calcium-dependent activation system involves an activator protein named calmodulin analogous to that described for other Ca^{2+}-dependent systems. Although the mechanism of activation has not yet been completely elucidated, Lynch and Cheung [11] suggest that Ca^{2+} induces the formation of a calmodulin-ATPase complex, ie, that in the presence of Ca^{2+}, the enzyme and calmodulin form an active holoenzyme. It is known that –SH reagents such as mercurials, dithionitrobenzoic acid, N-ethylmaleimide, diazenedicarboxylic acid N,N-dimethylamide (diamide) [11] can affect some of the erythrocyte membrane properties: deformability [12], permeability [13], enzyme activities [14, 15], etc. The efficiency of these substances and the reversibility of their action are strictly related to their permeability, binding capacities, and reactivities with either pairs of thiols or single thiols. In our "white membrane" preparations two divalent dependent ATPase activities can be evoked by adding either Ca^{2+} and Mg^{2+}, or Mg^{2+} alone. To investigate the role of the neighboring –SH groups in the mechanism of such ATPase activities, we have tested the effects of various divalent cation concentrations and of diamide, a mild oxidizing agent, acting on thiol groups close to each other, which easily crosses erythrocyte membranes and does not bind to them.

MATERIALS AND METHODS

Preparation of Red Cell Membranes

Outdated human blood in sterile bottles containing acid-citrate-dextrose was kindly donated by the Hospital blood bank (Padova, Italy). Erythrocytes were obtained by centrifugation at 1,600g for ten min at 2°C. The buffy coat was carefully removed by suction, and the cells were washed four times in 15 volumes of ice-cold medium containing 125 mM NaCl, and 30 mM Tris-HCl buffer, pH 7.4.

Hemoglobin-free membranes were prepared by a modification of the method of Blostein [16]: erythrocytes were lysed in 10 volumes of a medium containing Tris-HCl 5 mM and EDTA-Tris 1 mM, pH 7.7, centrifuged at 50,000g for 30 min, and the pellets were pooled and incubated at 0°C for 3 hours in EDTA-Tris 10 mM, pH 7.7. The membranes were then washed four times in 10 volumes of Tris-HCl, pH 7.7, pooled, made leaky by freezing and thawing, and stored at –20°C until used for ATPase activity and –SH content assays. Membrane protein determination was performed according to the method of Lowry et al [17], using crystalline bovine serum albumin as a standard.

ATPase Activity Assay

ATPase activity was assayed by measuring the inorganic phosphate produced, using a modification of the method of Fiske and Subbarow [18]. Incubations were carried out at 30°C under constant stirring, using 2 mg protein/ml suspension. The incubation medium, without added K^+, contained 77 mM NaCl, 11 mM Tris-HCl pH 7.4, 5 mM ouabain [1, 2], 5 mM Na_2-ATP, and the substances indicated in the figure legends. All the ATP solutions were neutralized with Tris base before use. At stated intervals aliquots of the membrane suspension were deproteinized with 15% (final concentration) trichloroacetic acid and centrifuged. One milliliter aliquots of the supernatant were then

taken for assay. Blanks were run to correct for nonenzymatic ATP hydrolysis and for the interference of DTE with inorganic phosphate assay. In spite of the standardization of the membrane preparation method, absolute total ATPase activity was not always constant in the different membrane pools due to the variability in age and quality of the blood samples.

For this reason, the results presented in this paper represent a typical set of experiments performed on a single membrane preparation, obtained from a single blood bottle. All assays were duplicated, and individual experiments were repeated four times. Thus each reported value is the mean of the values obtained in four experiments, which never differed from each other by more than 10%.

Membrane –SH Estimation

Incubations were carried out as described for the ATPase assay. One ml aliquots were centrifuged in a Beckman Microfuge model B and carefully washed many times in the same volume of the incubation medium to discard the diamide. Supernatants were assayed at 300 nm for diamide content. Membranes were then dissolved in 5% sodium dodecylsulphate (SDS) for a final concentration of 4 mg of protein/ml. Thiols were then assayed according to Ellman [19] in 0.2 M Tris-HCl buffer, pH 8.1, 5 mM EDTA; 10 mM 2,2′-dinitro-5,5′-dithiobenzoic acid (DTNB). The average total –SH content of our membrane preparations was about 55 nmoles/mg protein.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out in a Shandon apparatus at 20°C according to Fairbanks et al [20] without DTE in the buffer. The acrylamide concentration was 5.6% crosslinked with 0.21% N,N′-methylene-bis acrylamide. Proteins were stained with Coomassie brilliant blue.

RESULTS

By blocking of the Na^+-K^+-dependent ATPase (see medium composition in Materials and Methods), a low basal Mg^{2+}-ATPase activity can be detected in our white membranes in addition to the Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase. To assess whether EDTA buffers caused a selective protein release during membrane preparation, the protein composition of white membranes was tested by polyacrylamide gel electrophoresis in SDS. The gel pictures in Figure 1A, B, and C, when compared with the reports by other authors [21, 22] who used preparation buffers without EDTA, seem to exclude any important qualitative loss of the main protein bands. However, it is important to note that SDS-gel electrophoresis cannot exclude the possibility of a selective extraction of the activator protein by EDTA buffers, as described by other authors [23].

The Ca^{2+}-stimulated ATPase activity could be induced, in the presence of high Mg^{2+} concentrations, by the addition of increasing Ca^{2+} amounts. As shown in Figure 2A the K_{Ca} and the V_{maxCa}, as calculated from our results, are rather low, approximately 0.588 mM and 16.6 nmoles p_i/min/mg of protein, respectively.

The basal Mg^{2+}-ATPase activity can be evoked, in the absence of added Ca^{2+}, by the addition of increasing Mg^{2+} concentrations. For this activity a K_{Mg} of about 2 mM and a V_{max} of 3.2 nmoles P_i/mg protein/min have been obtained (Fig. 2B). It is important to add that Ca present as a contaminant did not exceed 1.6 nmoles/ml suspension as measured by atomic absorption and that at such a low Ca^{2+} concentration the (Ca^{2+} + Mg^{2+})-

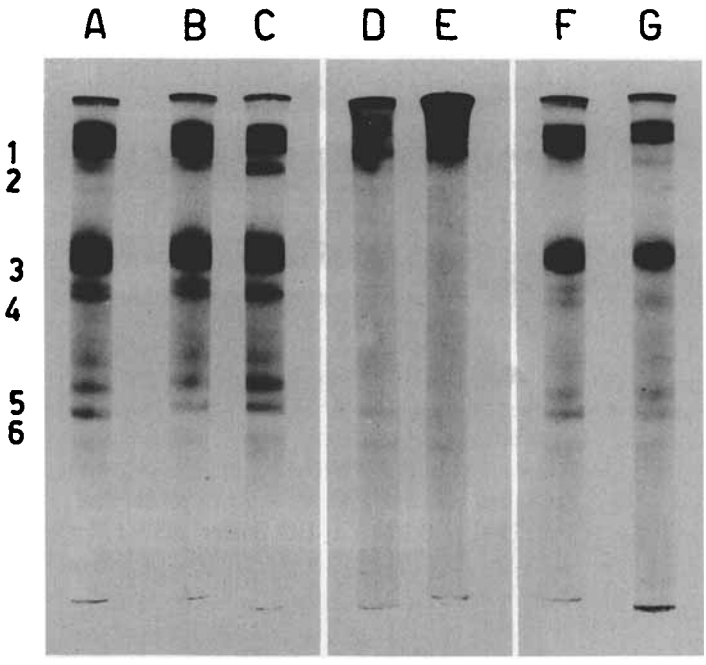

Fig. 1. Polyacrylamide gel electrophoresis of erythrocyte membrane proteins: effect of diamide. The numerical designation for the major polypeptides is adopted from Fairbanks et al [20]. All the samples were incubated for 30 min in the medium indicated in the Materials and Methods. A): No further additions. B): 10 mM $MgCl_2$; C): 10 mM $MgCl_2$ and 1 mM $CaCl_2$; D): 10 mM $MgCl_2$ and 2 mM diamide; E): 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 2 mM diamide; F): 10 mM $MgCl_2$, 2 mM diamide and 6 mM DTE; G): 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM diamide and 6 mM DTE. DTE when present, was added after 30 min incubation and the samples collected after another 10 min.

ATPase activation is rather unlikely. Moreover, the presence of a contaminating Ca^{2+}-dependent activation was also excluded by experiments in which 10 mM EGTA was added to the incubation mixture, causing no detectable decrease in the basal Mg^{2+}-ATPase activity (data not shown).

As shown in Figure 3, Ca^{2+} by itself is unable to evoke any ATPase activity in our white membranes, while a detectable activity does appear as soon as very low Mg^{2+} concentrations are added (Mg^{2+} present as a contaminant did not exceed 1.0 nmoles/ml suspension, as measured by atomic absorption).

Both ATPase activities, when supplemented with Ca^{2+}+Mg^{2+}, or Mg^{2+} concentrations able to induce their maximal rates, were inhibited by 2 mM diamide as shown in Table I. The diamide concentration was determined on the basis of the experiment reported in Figure 4, which indicates that 2 mM diamide is sufficient to produce (in 30 min at the protein concentration used) both the maximal oxidation of the membrane –SH groups (about 50% of the untreated membrane –SH content) and the maximal inhibition of the (Ca^{2+}+Mg^{2+})-ATPase activity. From the polyacrylamide gel pictures (Fig. 1D, E), it can be seen that the diamide action results in a dramatic change of the protein distribution leading to the appearance of a relevant portion of proteins at the top

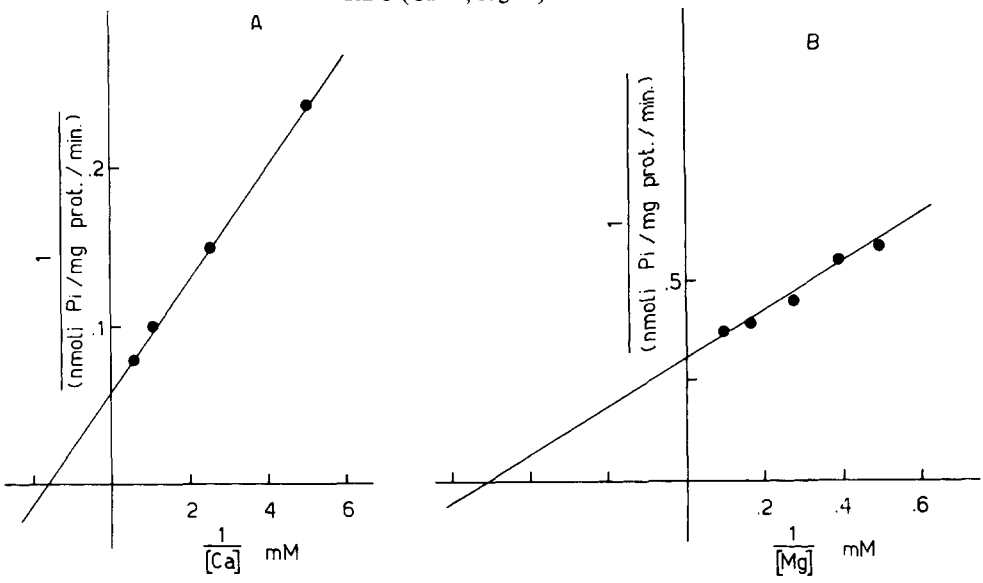

Fig. 2. A: K_{Ca} and V_{maxCa} of (Ca^{2+}+Mg^{2+})-ATPase in the presence of 10 mM Mg^{2+}. The activity is expressed as nmoles of inorganic phosphate/mg protein/min, and the incubation conditions are described in Materials and Methods. B: K_{Mg} and V_{maxMg} of Mg^{2+}-ATPase. The activity is expressed as nmoles of inorganic phosphate/mg protein/min, and the incubation conditions are described in Materials and Methods. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

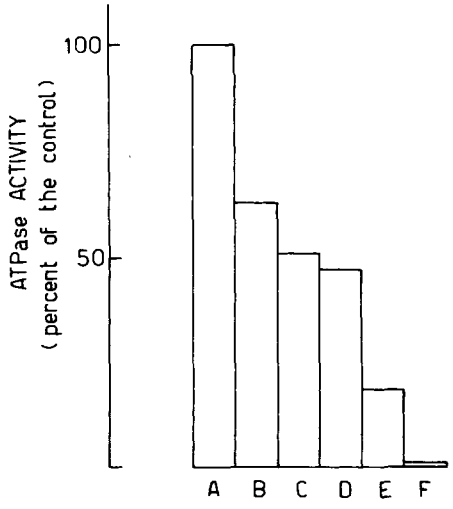

Fig. 3. Effect of different Ca^{2+}: Mg^{2+} ratios on the (Ca^{2+}+Mg^{2+})-ATPase activity. The incubation conditions are described in Materials and Methods. The activities are given as percent of the control and are calculated from the inorganic phosphate produced in the time interval 45–60 min during incubation. A: 0.2 mM Ca^{2+}, 0.5 mM Mg^{2+}; B: 0.15 mM Ca^{2+}, 0.75 mM Mg^{2+}; C: 0.5 mM Ca^{2+}, 0.1 mM Mg^{2+}; D: 0.1 mM Ca^{2+}, 1 mM Mg^{2+}; E; without Ca^{2+}, 10 mM Mg^{2+}; F: 1 mM Ca^{2+}, without Mg^{2+} Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

TABLE I. Total Membrane –SH Content After Diamide Action in the Presence and Absence of Ca^{2+} and Mg^{2+}

	Membranes $-Ca^{2+}$ $-Mg^{2+}$	Membranes $+Ca^{2}$ $+Mg^{2+}$	Membranes $+Mg^{2+}$ $-Ca^{2+}$	Membranes $-Mg^{2+}$ $+Ca^{2+}$
– DIAM	55.31	50.88	47.95	54.68
+ DIAM	37.34	22.07	24.66	22.66

The incubation medium contained, when indicated, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM diamide. The values are expressed as nmoles – SH/mg protein. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

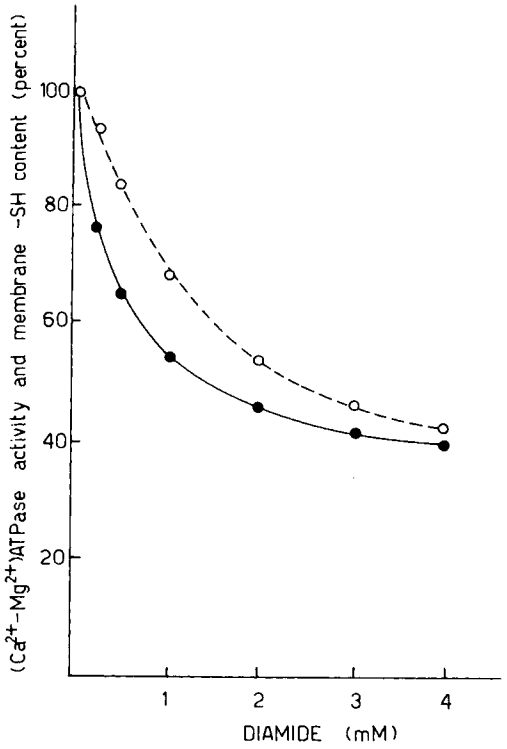

Fig. 4. Effect of diamide on the Ca^{2+}-Mg^{2+}-dependent ATPase activity (•—•) and total –SH content (○- - -○). Incubation medium contained 10 mM MgCl and 1 mM CaCl, and the conditions are described in Materials and Methods. Incubation time was 30 min. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

of the gel. Such a portion may be ascribed to protein aggregates produced by disulfide bond formation too large to enter the gel. Furthermore, no difference in the reagent efficiency seems to be evoked by the presence of both Ca^{2+} and Mg^{2+} alone during its action, according to the results of –SH assays reported later.

As can be observed in Figure 5, the ATPase activity measured in the presence of both Ca^{2+} and Mg^{2+} is decreased by addition of excess EGTA which, by chelating Ca^{2+}, inhibits the (Ca^{2+} +Mg^{2+})-ATPase while leaving the basal Mg^{2+}-activity unaffected. In the presence of diamide, the ratio between the rates of ATP hydrolysis observed both in the presence and in the absence of free Ca^{2+}, was about the same as in the absence of the reagent. This finding seems to indicate that the presence of diamide does not affect the efficiency of the (Ca^{2+} +Mg^{2+})-ATPase amplification system. The same conclusion can be reached from the data of Figure 6, which show the increase of the Mg^{2+}-dependent basal activity induced by Ca^{2+} addition in diamide-treated and untreated membranes.

Since, as indicated before, diamide is able to complete its action in less than 30 min, the main difference between the two reported experiments (Figs. 5 and 6) is represented by diamide exerting its action in the presence of both Ca^{2+} and Mg^{2+} in the former case, or of Mg^{2+} alone in the latter, and no significant difference in the diamide effect on the

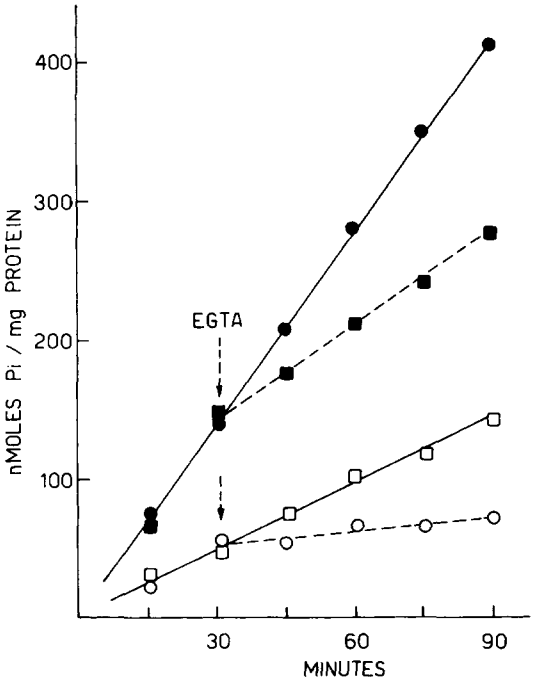

Fig. 5. Effect of EGTA on the (Ca^{2+} +Mg^{2+})-dependent ATPase in the presence and in the absence of diamide. (□—□) 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM diamide; (●—●) 10 mM $MgCl_2$, 1 mM $CaCl_2$; (■ ; ○) 10 mM EGTA added after 30 min incubation, as indicated by the arrows. The incubation conditions are described in Materials and Methods. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

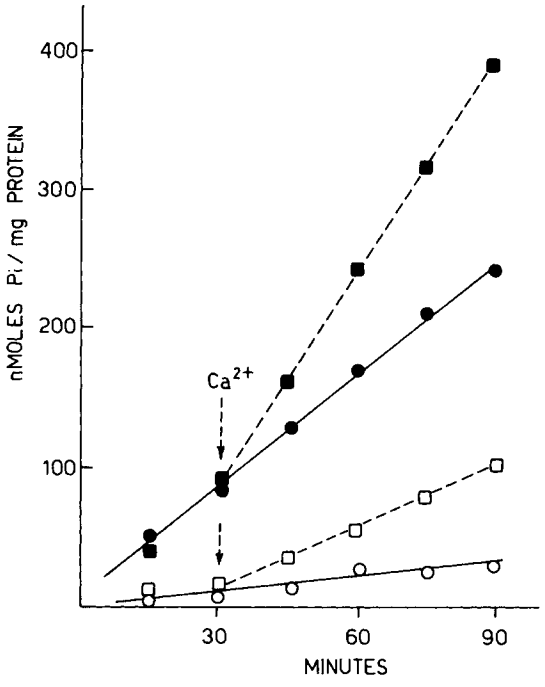

Fig. 6. Effect of Ca^{2+} on the Mg^{2+}-dependent ATPase activity in the presence and in the absence of diamide. (○—○) 10 mM $MgCl_2$, 2 mM diamide; (●—●) 10 mM $MgCl_2$; (■ ; □) 1 mM $CaCl_2$ added after 30 min incubation, as indicated by the arrows. The incubation conditions are described in Materials and Methods. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

ATPase activities was, however, observed in the two cases. These observations agree with the results reported in Figure 1 and in Table I where no significant difference can be observed between the relative amounts of total membrane thiols left unaffected by diamide in the presence of both Ca^{2+} and Mg^{2+} or Mg^{2+} alone.

Addition of DTE in large excess with respect to the diamide concentration was used to induce an overall thiol reduction in the membranes and to inactivate diamide (by reducing its diazene bond). The data reported in Table II indicate that 6 mM DTE caused a significant restoration of the ATPase activity of diamide-treated membranes in the presence of Ca^{2+} and Mg^{2+}, while no satisfactory reversal of diamide inhibition could be seen in the presence of Mg^{2+} alone, the maximum activity reached being less than 50% of the control.

The protein pattern in Figure 1F, G shows that DTE was able to restore the normal distribution of the main protein bands. However, we must point out that in the case of diamide plus DTE-treated membranes the protein bands after SDS-gel electrophoresis appeared lighter than in the control gels, and that a visible amount of material (although not evident from the gel pictures) still remained at the top of the gel.

TABLE II. ATP Splitting Rates in the Presence of Diamide and After DTE Treatment in the Presence of Both Ca^{2+} and Mg^{2+} or Mg^{2+} Alone

	Membranes $+Mg^{2+}$	Membranes $+Mg^{2+}+Ca^{2+}$
– DIAM – DTE	2.66	10
+ DIAM – DTE	0.5	5
+ DIAM + DTE	1.2	9.33

The incubation medium contained, when indicated in the table, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM diamide, 6 mM DTE. DTE was added after 30 min incubation with diamide, and the reported rates are calculated on the inorganic phosphate release as measured in the incubation interval 45–60 min. The values are expressed as nmoles P_i/mg protein/min. Each reported value is the mean of the values obtained in four experiments performed in duplicate. Values never differed from each other by more than 10%. All the experiments presented in this paper were performed on the same membrane preparation.

DISCUSSION

As reported in the Results section, both ATPase activities described in this paper have an absolute requirement for Mg^{2+}. If we consider that the proposed enzyme mechanism for the ($Ca^{2+}+Mg^{2+}$)-ATPase involves a stepwise process for ATP splitting [10] and a Ca^{2+}-dependent activation system (which may have the so-called calmodulin [11] as the most important factor) our results seem to implicate Mg^{2+} as an essential factor for the enzyme activity. In fact, when Mg^{2+} is present in concentrations sufficient to induce the ATP hydrolysis reaction sequence, the rate of the reaction increases parallel to the Ca^{2+} concentration until a maximum rate is reached. On the contrary, in the absence of sufficient Mg^{2+}, even realtively high Ca^{2+} concentrations can not induce any detectable activity.

It is relevant to note that our membranes were prepared in EDTA buffers (see Materials and Methods), which, according to the reports of Katz et al [23] may lead to membrane preparations devoided of activator protein. On the other hand, Lynch and Cheung [11] have demonstrated that erythrocyte membranes can not be completely depleted of calmodulin, even by treatment with such a strong Ca^{2+} chelator as EGTA. According to those authors, small amounts of calmodulin (100–200 ng/mg of membranes) able to produce a detectable ATPase activation remain bound to the membranes even after such treatment. This observation seems to reflect the condition of our membranes too, which exhibit a very low Ca^{2+} affinity (between 500 and 600 μM), in comparison with that usually exhibited by nontreated membranes (about 1 μM) [24, 25] and a concomitant low maximal rate of ATP splitting.

Diamide, which in intact cells may also form mixed disulfides between membrane and soluble thiols, in isolated membranes will react only with pairs of thiols close to each

other. The reported observations seems to agree with the point of view that the membrane perturbation induced by diamide primarily affects the stage of the ATP hydrolysis mechanism common to both ATPase activities.

In fact, the feeling is that the high efficiency of the amplification system is able to enhance a very low production of the phosphorylated intermediate [10, 26, 27]; or by inducing a more rapid liberation of inorganic phosphate, it may also stimulate the intermediate production, even under such particular conditions. This in spite of the very small amount of calmodulin that is probably present in the membranes and the evident membrane damage consequent to –SH couples oxidation.

In other words, in spite of the evident alterations shown in the electrophoretic patterns, the oxidation of neighboring thiols seems to leave largely unaffected the mechanism of the $(Ca^{2+}+Mg^{2+})$-ATPase amplification system evoked by Ca^{2+}.

Since the –SH groups of the particular ATPase complex conceivably represent only a minor fraction of the total membrane –SH able to react with diamide, the effect of diamide should reasonably be regarded as a consequence of some important modifications of the ATPase microenvironment produced by disulfide bridges formation. But results lead to the hypothesis that the Ca^{2+}-calmodulin-enzyme complex may be formed and that Ca^{2+} may play its role in the ATPase mechanism even in such an adverse condition. In the case of the Mg^{2+}-ATPase basal activity, Mg^{2+} may partially support all the stages of the process that leads to ATP splitting, but the efficiency of such a system appears to be so low that diamide inhibition reduces it to an undetectable level. As reported in the results section, the reduction of disulfides induced by DTE leads to the restoration of the ATPase activity in the presence of both Ca^{2+} and Mg^{2+}, while the restoration is less than 50% of the control rate in the presence of Mg^{2+} alone.

Thus the redox state of a couple of –SH groups close to each other and that are possibly related to the $(Ca^{2+}+Mg^{2+})$-ATPase active site, may be an important factor if Ca^{2+} is to function at optimal efficiency.

This is shown by DTE's effectiveness when Ca^{2+} along with Mg^{2+} is available to the enzyme and by its inability to fully restore the ATPase activity when Mg^{2+} alone does support all stages of the ATPase reaction sequence described by Rega and Garrahan [10]. In fact, no significant differences in the total membrane –SH content, and protein electrophoretic distribution can be observed in the two cases. However, it should be stressed that the reduction of some physiological disulfides may induce functional modifications of the membranes and that according to Triplett et al, Ca^{2+} per se is able to evoke some variations of the membrane protein distribution [21]. However, this does not seem to be true in our case, as demonstrated by the gel pictures. Finally, the Mg^{2+} function in the ATPase reaction sequence does not show a complete dependence on the redox state of membrane –SH groups that are close to each other, since diamide is unable to completely abolish the P_i release in the presence of Ca^{2+} stimulation; and, on the other hand, Ca^{2+} alone does not induce any inorganic phosphate release.

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