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LIGAND-INDUCED CONFORMATIONAL CHANGES IN THE (Mg^{2+} + Ca^{2+})-DEPENDENT ATPase OF RED CELL MEMBRANES

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SUMMARY

1. In the presence of Ca^{2+}, Mg^{2+} or Mg-ATP, the (Mg^{2+} + Ca^{2+})-dependent ATPase from human red cell membranes undergoes conformational changes. These changes result in an enhanced susceptibility of the enzyme to thermal inactivation, tryptic digestion or inhibition by the sulfhydryl reagent *N*-ethylmaleimide. Any one of these effects was taken as evidence of a conformational change.

2. Monovalent cations, in contrast to the other ligands, protected against tryptic digestion. They did not affect either the thermal stability of the enzyme or its reactivity toward *N*-ethylmaleimide.

3. Of the ligands tested, Ca^{2+} had the most pronounced effect as judged by all three criteria. With *N*-ethylmaleimide or trypsin as inactivating agents, Ca^{2+} specifically increased the rate of inactivation of (Mg^{2+} + Ca^{2+})-dependent ATPase and did not alter the stability of either Mg^{2+}- or (Mg^{2+} + Na^{+} + K^{+})-dependent ATPase. With heat as the inactivating treatment, Ca^{2+} increased the rate of inactivation of both Mg^{2+}- and (Mg^{2+} + Ca^{2+})-dependent ATPase. However, the effect on Mg^{2+}-dependent ATPase was less pronounced (60 % inactivation in 40 min at 47 °C) than that on (Mg^{2+} + Ca^{2+})-dependent ATPase (95 % inactivation in 40 min at 47 °C).

4. Ca^{2+} stimulated ATP hydrolysis and increased reactivity of the enzyme toward *N*-ethylmaleimide over a similar range of Ca^{2+} concentrations. All these observations, taken together, suggest that Ca^{2+} acted by binding at the active center of (Mg^{2+} + Ca^{2+})-dependent ATPase.

5. Mg^{2+} increased the susceptibility of the enzyme to tryptic digestion and to inhibition by *N*-ethylmaleimide, but in both cases produced a smaller effect than Ca^{2+}. This was true even when the Mg^{2+} concentration was 10–15 times higher than the Ca^{2+} concentration. Mg^{2+} had no effect on the thermal stability of the enzyme.

6. Mg-ATP increased the reactivity of the enzyme toward *N*-ethylmaleimide. Other criteria were not tested with respect to this ligand. Mg-ATP caused about the same increase in sulfhydryl reactivity as did Mg^{2+} alone.

7. When Mg-ATP and Ca^{2+} were present together, the sulfhydryl reactivity of the enzyme was intermediate between that with either ligand alone. Since the

Abbreviation: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

enzyme is active in the presence of these two ligands, and since a conformational change can be detected in their presence, it is suggested that conformational changes accompany activity in this enzyme, and that these conformational changes might be associated with the movement of Ca^{2+} during transport.

INTRODUCTION

The uptake of Ca^{2+} by sarcoplasmic reticulum and the extrusion of Ca^{2+} from human red cells are active processes which use ATP as a direct source of energy[1–4]. These transport systems appear in isolated membranes as (Mg^{2+} + Ca^{2+})-dependent ATPase activities which reflect the coupling of ATP hydrolysis to Ca^{2+} movement[1,4–6].

The (Mg^{2+} + Ca^{2+})-dependent ATPase activity of both systems is the resultant of at least two sequential reactions: (1) transfer of the terminal phosphate of ATP to the enzyme to yield a phosphoenzyme intermediate, and (2) hydrolysis of the phosphoenzyme to release inorganic phosphate[7,8]. In sarcoplasmic reticulum, Ca^{2+} stimulates the first reaction and Mg^{2+} the second[7]. In red cells, by contrast, the cation requirements for the two reactions appear to be the reverse[8]. The mechanism of (Mg^{2+} + Ca^{2+})-dependent ATPase thus resembles that of (Mg^{2+} + Na^+ + K^+)-dependent ATPase, at least to the extent that in both systems cations stimulate the formation and hydrolysis of a phosphoenzyme intermediate, and these reactions are coupled to cation transport[9]. The question of how this coupling is accomplished, however, remains unanswered.

The phosphoenzyme intermediate of (Mg^{2+} + Na^+ + K^+)-dependent ATPase appears to exist in at least two forms (or conformations), and it has been suggested that conformational changes interconverting these forms could lead to the transport of bound Na^+ and K^+ (ref. 10; see also refs 11 and 12). Recently Cha *et al.*[8] reported that the phosphoenzyme intermediate of red cell (Mg^{2+} + Ca^{2+})-dependent ATPase also exists in at least two forms, and shows a number of other similarities to that of (Mg^{2+} + Na^+ + K^+)-dependent ATPase. Thus there might be a conformational basis for transport in this system as well.

The experiments described here were designed to detect conformational changes in the (Mg^{2+} + Ca^{2+})-dependent ATPase of red cells, due to the binding of ligands which are required for activity (Mg^{2+}, Mg-ATP and Ca^{2+}). All three ligands were found to induce characteristic changes in conformation which resulted in an enhanced susceptibility of the enzyme to thermal inactivation, to tryptic digestion or to inhibition by *N*-ethylmaleimide. It is suggested that these conformational changes might reflect events in the normal reaction sequence of the enzyme, which are involved with Ca^{2+} movement.

METHODS

Preparation of ATPase

Outdated human blood was obtained from the blood bank, and the membranes were isolated as described previously[13]. The protein content of each preparation was measured by the method of Lowry *et al.*[14]. In some experiments, fresh blood was used

as the source of the enzyme, and the results were always consistent with those obtained from outdated blood.

ATPase assay

ATPase activity was assayed by measuring the inorganic phosphate released from ATP during 1–2 h incubations at 38 °C. Phosphate was measured as described previously[13] using a modification of the method of Fiske and SubbaRow[15]. Incubations were carried out in a volume of 2 ml, and all tubes contained 1.5 mM Tris–ATP, 1.7 mM Mg^{2+}, 60–120 mM Na^+ and 40 mM Tris–HCl buffer (pH 7.8). Other additions and modifications are given in the text. Na^+ was added to all tubes because Na^+ (or K^+) has been shown to stimulate ATPase activity in the presence of Mg^{2+} and Ca^{2+} (refs 13 and 16). All cations were added as their chloride salts. Di-sodium ATP (Sigma Chemical Co., St. Louis, Mo.) was converted to the Tris salt by passage through a chilled column of Dowex 50 X8-100 cation-exchange resin in the Tris form.

Mg^{2+}-dependent ATPase activity is the activity measured in the presence of Mg^{2+} and Na^+. The (Mg^{2+} + Na^+ + K^+)-dependent component of activity is calculated by subtracting the Mg^{2+}-dependent activity from the activity measured in the presence of Mg^{2+}, Na^+ and K^+. Similarly, the (Mg^{2+} + Ca^{2+})-dependent component of activity is calculated by subtracting the Mg^{2+}-dependent activity from the activity measured in the presence of Mg^{2+}, Ca^{2+} and Na^+.

Exposure of membranes to heat, N-ethylmaleimide and trypsin

Exposures were carried out in a medium containing 50 mM Tris–HCl buffer (pH 7.8). Other conditions are given below and in the text. Exposure was begun by adding membranes to flasks which contained all other ingredients. At timed intervals, aliquots were transferred to assay tubes on ice These tubes contained the complete medium for subsequent ATPase assay. After removal of the final aliquots from the flasks, these tubes were assayed for ATPase activity remaining.

Exposure to heat was carried out at 46–47 °C in a water-bath shaker.

Exposure to *N*-ethylmaleimide (0.1–0.5 mM) was carried out on ice. Assay tubes contained 2 mM 2-mercaptoethanol in this case, to inactivate the *N*-ethylmaleimide.

Exposure to trypsin (10^{-4} or $5 \cdot 10^{-5}$ mg/ml) was carried out at 38 °C in a water-bath shaker. The assay tubes contained $3 \cdot 10^{-4}$ mg of trypsin inhibitor in this case to inactivate the trypsin. Trypsin (Type III, 2 times crystallized, from bovine pancreas) and trypsin inhibitor (soybean, type 1-S) were obtained from Sigma.

When Ca^{2+} was present during exposure under any of these conditions, and Mg^{2+}- or (Mg^{2+} + Na^+ + K^+)-dependent activities were to be measured subsequently, the assay tubes contained 0.5 mM Tris EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid) to chelate the Ca^{2+}. This concentration of EGTA was found to be sufficient to prevent Ca^{2+} (0.10 mM) from stimulating (Mg^{2+} + Ca^{2+})-dependent ATPase and from inhibiting (Mg^{2+} + Na^+ + K^+)-dependent ATPase.

RESULTS

Effect of Ca^{2+} on the rate of thermal inactivation

In the experiment shown in Fig. 1, membranes were exposed at 47 °C with and without Ca^{2+}, and at the times indicated, aliquots were removed for assay of

Mg^{2+}- and (Mg^{2+} + Ca^{2+})-dependent ATPase activities. (Mg^{2+} + Ca^{2+})-dependent ATPase was quite stable when exposed without Ca^{2+}, but was rapidly inactivated when Ca^{2+} was present during exposure (see inset). The loss in activity at zero time in the presence of Ca^{2+} might have been due to: (1) the delay of 15–30 s between addition of the membranes and removal of the first aliquot, and (2) additional inactivation after removal of the first aliquot. The rate of thermal inactivation of Mg^{2+}-dependent ATPase was also increased by Ca^{2+}, but much less dramatically, and the effect was largely confined to the initial period of exposure (Fig. 1).

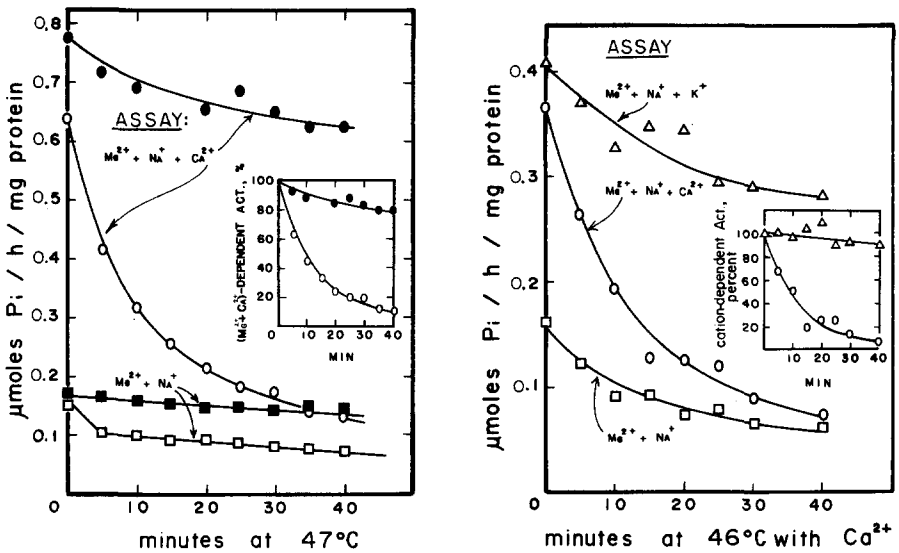

Fig. 1. Thermal inactivation of Mg^{2+}- and (Mg^{2+} + Ca^{2+})-dependent ATPase during exposure with and without Ca^{2+}. Membranes (0.26 mg protein/ml) were exposed at 47 °C as described in Methods with 0.06 mM Ca^{2+} (open symbols), and without Ca^{2+} (closed symbols). At the times shown, 1-ml aliquots were removed for assay of ATPase activity under the following conditions: □, ■, 120 mM Na^+ and 0.5 mM Tris EGTA; ○, ●, 120 mM Na^+ and 0.06 mM Ca^{2+}. Other conditions are given in Methods. Inset: (Mg^{2+} + Ca^{2+})-dependent component of activity expressed as a percent of the initial activity and calculated as described in Methods. ○, membranes exposed with Ca^{2+}; ●, membranes exposed without Ca^{2+}.

Fig. 2. Effect of heat on Mg^{2+}-, (Mg^{2+} + Ca^{2+})- and (Mg^{2+} + Na^+ + K^+)-dependent ATPase activities during exposure of membranes (0.30 mg protein/ml) at 46 °C in the presence of 0.06 mM Ca^{2+}. At the times shown, 1-ml aliquots were removed for assay of ATPase activity under the following conditions: □, 120 mM Na^+ and 0.5 mM Tris EGTA; ○, 120 mM Na^+ and 0.06 mM Ca^{2+}; △, 120 mM Na^+, 15 mM K^+ and 0.5 mM Tris EGTA. Other conditions are given in Methods. Inset: (Mg^{2+} + Ca^{2+})- and (Mg^{2+} + Na^+ + K^+)-dependent components of activity expressed as a percent of the initial activity and calculated as described in Methods. △, (Mg^{2+} + Na^+ + K^+)-dependent activity; ○, (Mg^{2+} + Ca^{2+})-dependent activity.

Fig. 2 compares the effect of exposure with Ca^{2+} on the thermal stabilities of the three ATPase activities of the red cell membrane. Mg^{2+}-dependent ATPase decreased about 60% in 40 min, and (Mg^{2+} + Ca^{2+})-dependent ATPase was about 95% inactivated over the same interval. (Mg^{2+} + Na^+ + K^+)-dependent ATPase, in contrast, was stable under these conditions.

This labilizing action was specific for Ca^{2+}. Mg^{2+} did not alter the thermal

stability of (Mg^{2+} + Ca^{2+})-dependent ATPase at concentrations as high as 1.5 mM, and monovalent cations were similarly without effect.

The concentration of Ca^{2+} used in these experiments was within the range which activates (Mg^{2+} + Ca^{2+})-dependent ATPase (Fig. 8a). The simplest interpretation of these results is that Ca^{2+} acted by binding specifically at the active center of this enzyme to yield an unstable conformation. A change of this kind, if extensive enough, could be demonstrable as an altered sensitivity to other inactivating treatments as well, and this was found to be the case.

Effect of Ca^{2+}, Mg^{2+} and monovalent cations on the rate of tryptic digestion

When membranes were exposed to trypsin without Ca^{2+} present, (Mg^{2+} + Ca^{2+})- and (Mg^{2+} + Na^+ + K^+)-dependent ATPases were about equally sensitive to tryptic attack, as measured by loss in ATPase activity with time of exposure. With Ca^{2+} present, however, the rate of loss of (Mg^{2+} + Ca^{2+})-dependent ATPase was increased specifically: Ca^{2+} did not modify the sensitivity of the other ATPase activities to trypsin (Fig. 3). The initial stimulation of (Mg^{2+} + Ca^{2+})-dependent ATPase shown in this figure was not found in all experiments with trypsin. All activities were stable in the absence of trypsin, or when trypsin inhibitor was present along with trypsin during exposure. The selectivity of this effect of Ca^{2+} argues against the possibility that Ca^{2+} simply activated the trypsin, and indicates that Ca^{2+} acted directly on (Mg^{2+} + Ca^{2+})-dependent ATPase to expose one or more trypsin-sensitive peptide bonds.

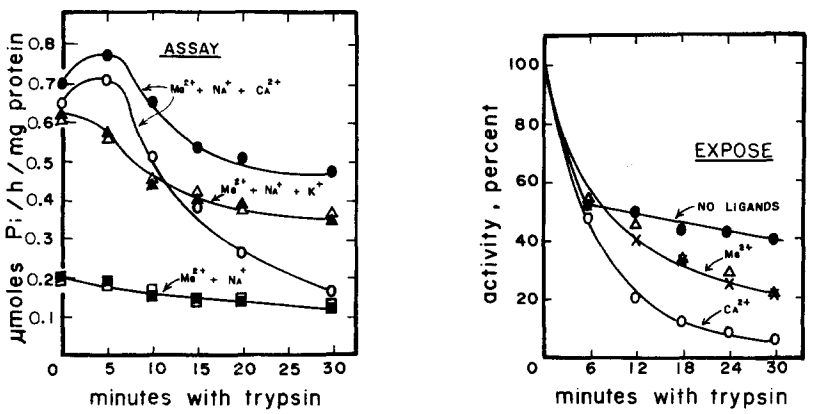

Fig. 3. Effect of trypsin on Mg^{2+}-, (Mg^{2+} + Ca^{2+})- and (Mg^{2+} + Na^{2+} + K^+)-dependent ATPase activities during exposure with and without Ca^{2+}. Membranes (0.70 mg protein/ml) were exposed to trypsin ($5 \cdot 10^{-5}$ mg/ml) at 38 °C with 0.1 mM Ca^{2+} (open symbols) and without Ca^{2+} (closed symbols). At the times shown, 0.5-ml aliquots were removed for assay of ATPase activity under the following conditions: □, ■, 100 mM Na^+ and 0.5 mM Tris EGTA; ○, ●, 100 mM Na^+ and 0.1 mM Ca^{2+}; △, ▲, 100 mM Na^+, 15 mM K^+ and 0.5 mM Tris EGTA. All assay tubes contained $3 \cdot 10^{-4}$ mg of trypsin inhibitor. Other conditions are given in Methods.

Fig. 4. Comparison of the effects of Mg^{2+} and Ca^{2+} on the trypsin susceptibility of (Mg^{2+} + Ca^{2+})-dependent ATPase. In this experiment, membranes (0.70 mg protein/ml) were exposed to trypsin ($1 \cdot 10^{-4}$ mg/ml) in the presence of: ●, no ligands; ○, 0.1 mM Ca^{2+}; ×, 0.1 mM Mg^{2+}; △, 1.0 mM Mg^{2+}. At the times shown, 0.5-ml aliquots were removed for assay of ATPase activity in the presence of 60 mM Na^+, 0.1 mM Ca^{2+} and 1.7 mM Mg^{2+} in all cases. Other activities were not measured. Activity is expressed as a percent of the zero time value which was 0.80 μmole P_i/h per mg protein. This figure represents the average of two experiments. Other conditions are given in Methods.

Mg^{2+} also increased the sensitivity of (Mg^{2+} + Ca^{2+})-dependent ATPase to trypsin, but was less effective than Ca^{2+} (Fig. 4). This figure shows the total activity measured in the presence of Mg^{2+} and Ca^{2+}. Mg^{2+}-dependent ATPase was not affected by the variables of this experiment, and thus the changes reflect changes in (Mg^{2+} + Ca^{2+})-dependent ATPase.

Monovalent cations protected (Mg^{2+} + Ca^{2+})-dependent ATPase from tryptic attack, both in the presence and absence of Ca^{2+}. Fig. 5 shows this effect with Ca^{2+} present. Somogyi[17] reported that Na^+ and K^+ also protect (Mg^{2+} + Na^+ + K^+)-dependent ATPase from tryptic attack, and in his experiments, as in ours, K^+ appeared to be more effective than Na^+.

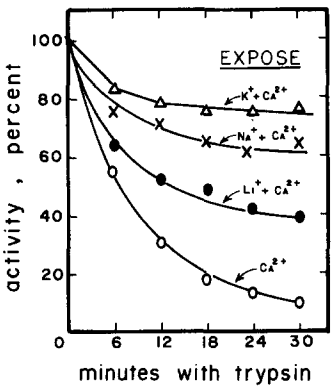

Fig.5. Effect of monovalent cations on the trypsin susceptibility of (Mg^{2+} + Ca^{2+})-dependent ATPase in the presence of Ca^{2+}. Membranes (0.70 mg protein/ml) were exposed to trypsin ($1 \cdot 10^{-4}$ mg/ml) in the presence of 0.1 mM Ca^{2+} in all cases. Other conditions for exposure to trypsin: ○, no additional ligands; △, 60 mM K^+; ×, 60 mM Na^+; ●, 60 mM Li^+. At the times shown, 0.5-ml aliquots were removed for ATPase assay in the presence of 0.1 mM Ca^{2+}, 60 mM Na^+, 15 mM K^+, 15 mM Li^+ and 0.25 mM ouabain. This combination of monovalent cations allowed assay of all tubes under identical conditions, and ouabain prevented the appearance of (Mg^{2+} + Na^+ + K^+)-dependent ATPase activity. Activity is expressed as a percent of the zero time value which was 0.72 μmole P_i/h per mg protein. This figure represents the average of two experiments. Other conditions are given in Methods.

Effect of Ca^{2+}, Mg^{2+} and Mg-ATP on inhibition by N-ethylmaleimide

Wins and Schoffeniels[5] reported that (Mg^{2+} + Ca^{2+})-dependent ATPase from red cells was selectively inhibited by the sulfhydryl group reagent, salyrgan. We chose *N*-ethylmaleimide as the sulfhydryl reagent of choice because its effects on (Mg^{2+} + Na^+ + K^+)-dependent ATPase have been studied by others[18–20], and this afforded the possibility of making comparisons with previous work. At 38 °C, *N*-ethylmaleimide rapidly inactivated (Mg^{2+} + Ca^{2+})-dependent ATPase at concentrations (10^{-5} M) which had little or no effect on the other ATPase activities. A measurable rate of inhibition was obtained by exposing membranes to *N*-ethylmaleimide (0.1–0.5 mM) on ice. The effect on all three ATPase activities of exposing membranes under these conditions, with and without Ca^{2+}, is shown in Fig. 6. Ca^{2+} specifically increased the reactivity of (Mg^{2+} + Ca^{2+})-dependent ATPase toward *N*-ethylmaleimide. There was a slight loss of Mg^{2+}-dependent ATPase, but this was not affected by Ca^{2+}, and (Mg^{2+} + Na^+ + K^+)-dependent ATPase was completely stable. Even at 38 °C and in the presence of 0.5 mM *N*-ethylmaleimide, where the latter enzyme was 50 %

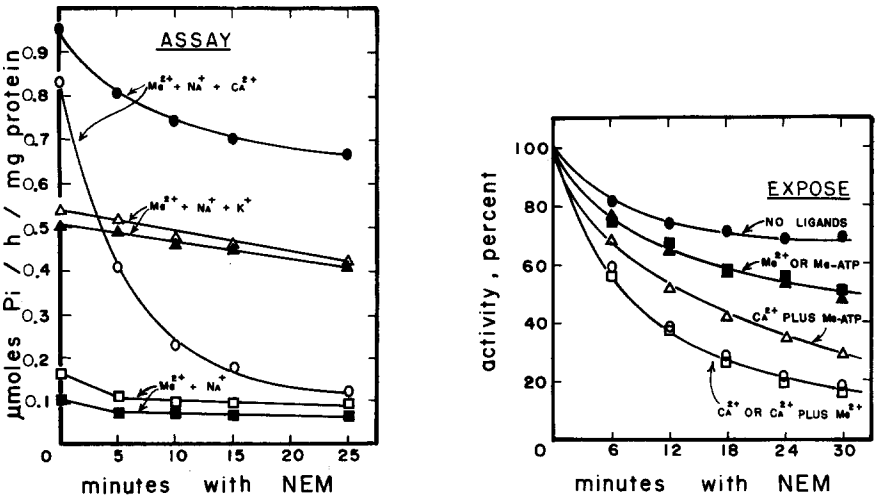

Fig. 6. Effect of *N*-ethylmaleimide (NEM) on Mg^{2+}-, (Mg^{2+} + Na^+ + K^+)- and (Mg^{2+} + Ca^{2+})-dependent ATPase activities during exposure with and without Ca^{2+}. Membranes (0.30 mg protein/ml) were exposed to 0.5 mM *N*-ethylmaleimide on ice in the presence of 0.08 mM Ca^{2+} (open symbols) and without Ca^{2+} (closed symbols). At the times shown, 1-ml aliquots were removed for ATPase assay under the following conditions: □, ■, 80 mM Na^+ and 0.5 mM Tris EGTA; ○, ●, 80 mM Na^+ and 0.08 mM Ca^{2+}; △, ▲, 80 mM Na^+, 15 mM K^+ and 0.5 mM Tris EGTA. Other conditions are given in Methods.

Fig. 7. Effect of Mg^{2+}, Mg-ATP and Ca^{2+} on the reactivity of (Mg^{2+} + Ca^{2+})-dependent ATPase toward *N*-ethylmaleimide (NEM). Membranes (0.30 mg protein/ml) were exposed to 0.124 mM *N*-ethylmaleimide on ice in the presence of: ●, no ligands; ■, 1.5 mM Mg^{2+}; ▲, 1.5 mM Mg-ATP; ○, 0.1 mM Ca^{2+}; □, 1.5 mM Mg^{2+} and 0.1 mM Ca^{2+}; △, 1.5 mM Mg-ATP and 0.1 mM Ca^{2+}. At the times shown, 1-ml aliquots were removed for assay of ATPase activity in the presence of 60 mM Na^+ and 0.1 mM Ca^{2+}. Other conditions are given in Methods. Mg^{2+}-dependent activity was not assayed. Activity is expressed as a percent of the zero time value which was 0.70 μmoles P_i/h per mg protein. This figure represents the average of 4 experiments.

inhibited in 30 min, we found that Ca^{2+} did not increase the rate of inhibition. Thus the binding of Ca^{2+} to (Mg^{2+} + Ca^{2+})-dependent ATPase appears to cause a conformational change which exposes one or more essential sulfhydryl groups in this enzyme only.

The fact that experiments with *N*-ethylmaleimide could be carried out at 0 °C allowed us to study the effects of exposure to Mg-ATP without significant hydrolysis. Fig. 7 shows that Mg^{2+} and Mg-ATP both increased the reactivity of (Mg^{2+} + Ca^{2+})-dependent ATPase toward *N*-ethylmaleimide, although the concentrations required were higher than for Ca^{2+}, and the effects were less pronounced. These ligands had no effect on Mg^{2+}-dependent ATPase under the conditions of this experiment, and thus the effects seen are due to changes in the (Mg^{2+} + Ca^{2+})-dependent component of activity. In addition, these ligands did not alter the stability of the enzyme in the absence of *N*-ethylmaleimide. ATP alone protected slightly against inhibition by *N*-ethylmaleimide (data not shown). Skou and Hilberg[18] found that ATP has a similar protective effect on (Mg^{2+} + Na^+ + K^+)-dependent ATPase, but apparently they did not study the effect of Mg-ATP on the sulfhydryl reactivity of this enzyme. Although Mg^{2+} and Mg-ATP appeared equally effective at similar concentrations, there was a difference which was reflected in the response of the

enzyme to Ca^{2+} in the presence of these ligands: Mg-ATP reduced the effect of Ca^{2+} whereas Mg^{2+} did not. In other experiments, not shown, we found that this interference by Mg-ATP could not be overcome by a 4-fold increase in the Ca^{2+} concentration. This appears to eliminate the possibility that Mg-ATP simply competed with Ca^{2+} for binding to the enzyme, or that chelation of Ca^{2+} by ATP was a significant factor under the conditions of this experiment.

If Ca^{2+} binds specifically to the active center of (Mg^{2+} + Ca^{2+})-dependent ATPase to cause this conformational change, the dependence on Ca^{2+} concentration might be similar to that for stimulation of ATPase activity. These concentration dependencies are compared in Figs 8a and 8b with *N*-ethylmaleimide as the inactivating agent. (Mg^{2+} + Ca^{2+})-dependent ATPase had an apparent K_m for Ca^{2+} of 0.02 mM (Fig. 8a). By comparison the Ca^{2+} concentration giving a half-maximal rate of inhibition by *N*-ethylmaleimide was between 0.005 and 0.01 mM (Fig. 8b). A similar result was obtained in another experiment in which the rate of thermal inactivation was studied as a function of Ca^{2+} concentration. The sigmoid nature of the curve in Fig. 8b might or might not be of significance. The point to be made from these experiments, however, is simply that the concentration dependencies for the two processes are similar. The lack of better agreement between the two curves could be due to the different experimental conditions in the two cases (*e.g.* the temperature during exposure in Fig. 8b was 0 °C, and Mg-ATP was not present).

The K_m which we found for Ca^{2+} in activating (Mg^{2+} + Ca^{2+})-dependent

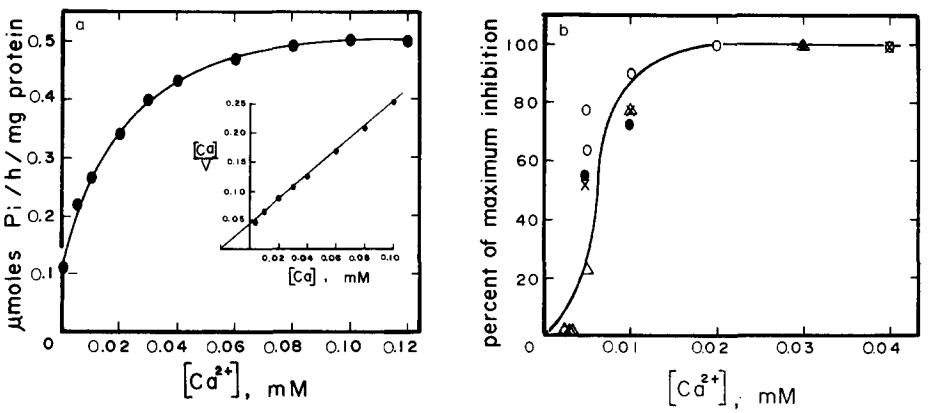

Fig. 8a. Effect of Ca^{2+} concentration on (Mg^{2+} + Ca^{2+})-dependent ATPase activity. The zero Ca^{2+} tube contained 0.5 mM Tris EGTA to chelate traces of Ca^{2+} which may have been present in the membranes. This point thus gives the Mg^{2+}-dependent activity. All tubes contained 60 mM Na^+ and 0.30 mg of membrane protein. Other conditions are given in Methods. Inset: data plotted according to a linear form of the Michaelis–Menten equation. v is the (Mg^{2+} + Ca^{2+})-dependent component of activity, calculated as described in Methods. The abscissa intercept gives the apparent K_m for Ca^{2+}. b. Effect of Ca^{2+} concentration on the rate of inhibition of (Mg^{2+} + Ca^{2+})-dependent ATPase by *N*-ethylmaleimide. Membranes (0.30 mg protein/ml) were exposed to *N*-ethylmaleimide (0.4 mM) in the absence of Ca^{2+} (control) and in the presence of the Ca^{2+} concentrations shown on the abscissa. At the times shown, 1-ml aliquots were removed and ATPase activity was subsequently measured as described in the legend to Fig. 7. The Ca^{2+}-dependent component of inhibition was taken as the difference (at 10 min) between the control and the various Ca^{2+}-containing samples. The difference at 0.03 mM Ca^{2+} was maximal and was taken as 100% for comparison with other differences. The activity at zero time was 0.72 µmole P_i/h per mg protein, and the control was inhibited by 25% at 10 min. The different symbols refer to different experiments.

ATPase is close to values found by Wins and Schoffeniels[5] and by Dunham and Glynn[21], but is about 5 times higher than values found by Schatzman and Rossi[16] and by Vincenzi[22]. The discrepancy may lie in the fact that the latter authors used a Ca^{2+} buffer to estimate free Ca^{2+} concentrations, whereas the former authors, and ourselves, reported only total Ca^{2+} concentrations. This would over-estimate the free Ca^{2+} concentration if a substantial fraction of the Ca^{2+} added were bound non-specifically to membranes and to ATP.

DISCUSSION

Low concentrations of Ca^{2+} induced a conformational change in (Mg^{2+} + Ca^{2+})-dependent ATPase which was seen as an increased susceptibility of the enzyme to thermal inactivation, to tryptic digestion and to inhibition by *N*-ethylmaleimide. The fact that this conformational change was revealed by three criteria, suggests that it involves a substantial re-orientation of the enzyme. This could be associated with a movement of bound Ca^{2+} to at least partially explain the transport function of this system.

This effect of Ca^{2+} was largely confined to (Mg^{2+} + Ca^{2+})-dependent ATPase. In none of the experiments did Ca^{2+} alter the stability of (Mg^{2+} + Na^+ + K^+)-dependent ATPase. The Mg^{2+}-dependent enzyme was more sensitive to thermal inactivation with Ca^{2+} present, but its reactivity toward trypsin and *N*-ethylmaleimide was unaffected by Ca^{2+}. Because Ca^{2+} acted with this degree of selectivity on (Mg^{2+} + Ca^{2+})-dependent ATPase, it is reasonable to assume that it acted by binding to its specific site at the active center of the enzyme. This assumption is additionally supported by the observation that Ca^{2+}-stimulated enzymatic activity and enhanced reactivity toward *N*-ethylmaleimide over a similar range of concentrations. Thus the conformational change caused by Ca^{2+} might reflect an event occurring normally when Ca^{2+} binds to the enzyme under conditions where the enzyme is active.

Monovalent cations had no effect on the thermal stability of (Mg^{2+} + Ca^{2+})-dependent ATPase or on its reactivity toward *N*-ethylmaleimide. They did, however, protect against tryptic attack. Somogyi[17] reported that Na^+ and K^+ have a similar protective effect on (Mg^{2+} + Na^+ + K^+)-dependent ATPase, and suggested that this might be due to a conformational change resulting from binding of these cations to specific sites on the enzyme. It is of interest that either Na^+ or K^+ alone activates (Mg^{2+} + Ca^{2+})-dependent ATPase, and saturation kinetics suggests that these cations act by binding to the enzyme[13,16]. Li^+ has, at best, only a slight stimulatory effect[13,16], and correspondingly afforded the least protection in our experiments (Fig. 5). The results of Somogyi[17] and ours could be easily explained on the assumption that monovalent cations simply inhibit trypsin, but Somogyi[17] presented evidence against this.

What we have referred to simply as (Mg^{2+} + Ca^{2+})-dependent ATPase activity actually includes a Na^+-dependent component which amounts to about 35 % of the total, since Na^+ was present in all assays. Schatzman and Rossi[16] have suggested that the Na^+-dependent and Na^+-independent components of (Mg^{2+} + Ca^{2+})-dependent ATPase might be different enzymes. This introduces a complication in the interpretation of our results, since we did not distinguish between these components in any of our experiments, but always measured their sum. However, considering

those experiments in which 90% or more of (Mg^{2+} + Ca^{2+})-dependent ATPase was inactivated, it is clear that both components must have been affected. Furthermore, in some preliminary experiments in which *N*-ethylmaleimide was used as the inactivating agent, we have found that the Na^+-dependent and Na^+-independent components of activity are inactivated at about the same rate in the presence of several ligands.

Mg^{2+} increased the reactivity of (Mg^{2+} + Ca^{2+})-dependent ATPase toward trypsin and *N*-ethylmaleimide, but was less effective than Ca^{2+}. By contrast, Mg^{2+} had no effect on the thermal stability of the enzyme. Thus the conformational change with Mg^{2+} is either qualitatively different from that with Ca^{2+}, or is simply not sufficient to be reflected in an altered thermal stability. These effects of Mg^{2+} could be due to binding to a site which is specific for Mg^{2+} (or Mg-ATP), or to the site which normally binds Ca^{2+}. The experiment shown in Fig. 7 argues against this latter possibility, however. If Mg^{2+} and Ca^{2+} acted at the same site, the sulfhydryl reactivity of the enzyme with Mg^{2+} and Ca^{2+} together should have been less than with Ca^{2+} alone, but this was not found to be the case.

Mg-ATP enhanced the reactivity of the enzyme toward *N*-ethylmaleimide and was as effective as Mg^{2+} alone, suggesting that these ligands bind to the same site to produce a similar result. Differences between these ligands became apparent, however, when they were added in combination with Ca^{2+}. With Mg^{2+} and Ca^{2+} the sulfhydryl reactivity of the enzyme was the same as with Ca^{2+} alone, whereas with Mg-ATP and Ca^{2+} the sufhydryl reactivity was intermediate between that with either ligand alone. For reasons mentioned in Results, this does not appear due to competition between Mg-ATP and Ca^{2+}. Furthermore, competition between Mg^{2+} and Ca^{2+} would seem a more likely possibility, and this was not found. This result could be explained most easily by assuming that the red cell enzyme is phosphorylated in the presence of Mg-ATP and Ca^{2+}, as occurs in sarcoplasmic reticulum, and that the phosphoenzyme has a lower sulfhydryl reactivity than the dephosphoenzyme. However, in a recent abstract, Cha *et al.*[8] reported that the red cell enzyme is phosphorylated in the presence of Mg-ATP, and that Ca^{2+} stimulates hydrolysis of the phosphoenzyme. This is in contrast to what occurs in sarcoplasmic reticulum, and it will be of interest to see if this difference in mechanism between the two systems is borne out in subsequent experiments. Regardless of mechanism, however, the fact that a conformational change could be detected in the presence of both Mg-ATP and Ca^{2+} is of interest, since the enzyme is active under these conditions. Thus conformational changes appear to accompany activity in this system and these conformational changes could be responsible for the movement of Ca^{2+}. The conformational changes seen in the presence of single ligands might reflect separate steps in the normal reaction sequence of the enzyme which lead to activity only when they occur in the proper order.

Skou and Hilberg[18] reported that Na^+, K^+ and ATP induced conformational changes in (Mg^{2+} + Na^+ + K^+)-dependent ATPase which modified sulfhydryl reactivity. More recently, Krupka[23] found that glucose and other sugars which are transported by the sugar transport system of the human red cell, induce conformational changes which are associated with an increased rate of inactivation of the system by 1-fluoro-2,4-dinitrobenzene. In both cases it was suggested that transport and the accompanying conformational change might be closely related events.

Since Ca^{2+} caused a conformational change even in the absence of Mg-ATP, it is clear that Ca^{2+} can bind to the dephosphoenzyme. If Ca^{2+} binds to the phosphoenzyme as well, it thus appears that the binding site is not the phosphate group itself, unless Ca^{2+} is transferred to this site secondarily. If Ca^{2+} were bound to the phospho- and dephospho-forms of the enzyme with different affinities, one necessary criterion for a system mediating active transport would be fulfilled: that there be two forms of a carrier with different affinities for the transported solute.

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