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Activation of the Human Red Cell Calcium ATPase by Calcium Pretreatment

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Abstract. Some kinetic parameters of the human red cell Ca^{2+}-ATPase were studied on calmodulin-free membrane fragments following preincubation at 37°C. After 30 min treatment with EGTA (1 mM) plus dithioerythritol (1 mM), a V_{max} of about 0.4 μmol P_i/mg × hr and a K_s of 0.3 μM Ca^{2+} were found. When Mg^{2+} (10 mM) or Ca^{2+} (10 μM) were also added during preincubation, V_{max} but not K_s was altered. Ca^{2+} was more effective than Mg^{2+}, thus increasing V_{max} to about 1.3 μmol P_i/mg × hr. The presence of both Ca^{2+} and Mg^{2+} during pretreatment decreased K_s to 0.15 μM, while having no apparent effect on V_{max}. Conversely, addition of ATP (2 mM) with either Ca^{2+} or Ca^{2+} plus Mg^{2+} increased V_{max} without affecting K_s. Preincubation with Ca^{2+} for periods longer than 30 min further increased V_{max} and reduced K_s to levels as low as found with calmodulin treatment.

The Ca^{2+} activation was not prevented by adding proteinase inhibitors (iodoacetamide, 10 mM; leupeptin, 200 μM; pepstatin A, 100 μM; phenylmethanesulfonyl fluoride, 100 μM). The electrophoretic pattern of membranes preincubated with or without Mg^{2+}, Ca^{2+} or Ca^{2+} plus Mg^{2+} did not differ significantly from each other. Moreover, immunodetection of Ca^{2+}-ATPase by means of polyclonal antibodies revealed no mobility change after the various treatments.

The above stimulation was not altered by neomycin (200 μM), washing with EGTA (5 mM) or by both incubating and washing with delipidized serum albumin (1 mg/ml), or omitting dithioerythritol from the preincubation medium. On the other hand, the activation elicited by Ca^{2+} plus ATP in the presence of Mg^{2+} was reduced 25–30% by acridine orange (100 μM), compound 48/80 (100 μM) or leupeptin (200 μM) but not by dithio-bis-nitrobenzoic acid (1 mM).

The fluorescence depolarization of 1,6-diphenyl- and 1-(4-trimethylammonium phenyl)-6-phenyl 1,3,5-hexatriene incorporated into membrane fragments was not affected after preincubating under the different conditions.

The results show that proteolysis, fatty acid production, an increased phospholipid metabolism or alteration of membrane fluidity are not involved in the Ca^{2+} effect. Ca^{2+} preincubation may stimulate the Ca^{2+}-ATPase activity by stabilizing or promoting the E_1 conformation.

Key words: Red cell Ca^{2+}-ATPase — Activation of Ca^{2+}-ATPase — Ca^{2+} action on Ca^{2+}-ATPase — Persistent Ca^{2+}-ATPase activation

Introduction

During the catalytic cycle, the human red cell Ca^{2+}-ATPase undergoes alternate conformational changes, displaying different affinities for Ca^{2+} and ATP [16, 17, 32, 33]. Ion translocation seems to arise from the large affinity drop of Ca^{2+} binding sites that accompanies the E_1-E_2 transition. Ca^{2+} movement, ATPase activity and conformational changes in the human red cell Ca^{2+} pump appear to be tightly coupled. Thus, it is possible to obtain net ATP synthesis by reversing the E_2-E_1 transition [38].

Early work provided evidence for conformational changes in the Ca^{2+}-ATPase following incubation with Ca^{2+} or Mg^{2+} [7]. As E_1 or E_2 conformers can be selectively sequestered by the appropriate ligands [17, 21], an alteration of the enzyme's kinetic parameters is expected after pretreatment with above cations. Thus, recent work on CaM-free[1] membrane fragments showed

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[1] Abbreviations: CaM, calmodulin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; K_s, apparent stability constant; DTE, dithioerythritol; DTNB, 5-5′-dithiobis(2-nitro)benzoic acid; PMSF, phenylmethanesulfonyl fluoride; DPH, 1,6-diphenyl 1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium phenyl)-6-phenyl 1,3,5-hexatriene; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

that the human red cell Ca^{2+}-ATPase behaves like a CaM-stimulated enzyme when it is pretreated with Mg^{2+} and vanadate [27]. Similarly, preincubation of the enzyme with Ca^{2+} ions in the millimolar range also results in an increased activity [2, 15, 30].

If the changes above do involve the respective conformer-ligand interactions, and assuming that the E_1 conformer has a K_s (Ca^{2+}) of about 1 μM in the absence of CaM [31], micromolar Ca^{2+} levels should be stimulatory after preincubation. To test this idea and also to confirm and extend previous findings, we have studied the effect of preincubation with Ca^{2+} at micromolar concentrations on some kinetic parameters of the human red cell Ca^{2+}-ATPase. It was found that the pretreated enzyme resembles a CaM-stimulated ATPase.

Materials and Methods

All reagents were of analytical quality whenever possible, mainly obtained from Sigma Chemical (St. Louis, MO) and The British Drug Houses, England.

Fresh O(+) blood from healthy human donors was used. CaM-free membrane fragments were prepared as described elsewhere [26] and stored at −80°C for not more than 30 days.

Preincubation of the fragments was done for 30 min at 37°C, in the presence of (mM): 130 KCl, 0.1 ouabain, 1 EGTA and 20 Tris-HCl (pH 7.55 at room temperature), with and without 1 DTE and the additions specified below. Thereafter, the fragments were washed twice with an ice-cold medium consisting of 140 mM KCl + 10 mM Tris-HCl (pH 7.55). The fragments were finally resuspended in a small volume of washing medium to obtain about 10 mg protein/ml.

ATPase Assays

The Ca^{2+}-ATPase activity of the incubated membranes was determined after 2 hr incubation at 37°C in a medium containing (mM): KCl, 130; $MgCl_2$, 2; EGTA, 1; ouabain, 0.1; Tris-HCl, 10 (pH 7.55 at room temperature) and different $CaCl_2$ concentrations set up to get free Ca^{2+} levels varying between 1–10 μM. The required amounts of Ca^{2+} and EGTA were calculated using a computer program [12]. The Ca^{2+}-ATPase activity was obtained by subtracting the activity found in the absence of Ca^{2+} from that in its presence. This activity was linear with time for up to two hours incubation, and it is expressed as μmol P_i/mg protein × hr.

Membrane lipid fluidity was assessed by monitoring the steady-state fluorescence depolarization of DPH and TMA-DPH at 37°C [34]. The probes were incorporated by preincubating membrane fragments in the presence of either fluorophore at 100 μM, as described earlier [27]. Fluorescence measurements were done on a SLM polarization fluorimeter (Champaign, IL) and corrected for light scattering effects [19]. The corrections never exceeded 5%. An excitation wavelength of 359 nm was used.

SDS-Polyacrylamide Gel Electrophoresis

Membrane fragments preincubated as above, were subjected to electrophoresis essentially as described by Laemmli [18], using 7.5% polyacrylamide gels. Proteins were stained with Coomassie blue and the electrophoretic patterns were evaluated by densitometry on a Bio-Rad video densitometer (model 620).

Immunodetection of Ca^{2+}-ATPase was done by conventional two-stage immunoblotting techniques [37], using polyclonal antibodies raised against the purified enzyme. The peroxidase-coupled anti-IgG was revealed with diaminobenzidine.

P_i determinations were done by a modification of the Fiske and Subbarow method [13], using $FeSO_4$ as reducing agent. Proteins were determined by the Lowry method [20], using bovine serum albumin as standard.

Statistical significance was assessed using Student's *t*-tests. Kinetic parameters were derived from linear regression analyses of Eadie-Hofstee plots, using a GPAD INPLOT4 software. r^2 values were always greater than 0.996.

Results

The Ca^{2+}-ATPase presented a relatively low Ca^{2+} affinity (K_s about 0.3 μM) and low V_{max} (0.4 μmol P_i/mg × hr) state after preincubation for 30 min with EGTA (1 mM) and DTE (1 mM) (Fig. 1). These values were almost identical to those found with nonincubated fragments (Table 1).

In contrast to previous findings [27], adding Mg^{2+} (10 mM) to the preincubation medium raised V_{max} (in μmol P_i/mg × hr) to nearly 1 (Fig. 1), a highly statistically significant increase ($P < 0.005$) (Table 1). A similar effect was also elicited by Ca^{2+}, bringing V_{max} to about 1.4 after preincubating with 10 μM Ca^{2+} (Table 1, Fig. 1). The minimal free Ca^{2+} concentration required for half-maximal activation was 0.5 μM, and stimulation was maximal at 1 μM (average results from two experiments).

Preincubating for 30 min with either divalent cation did not alter K_s significantly. However, K_s was reduced by half in the presence of both cations (Table 1). This change was highly statistically significant ($P < 0.005$).

The Ca^{2+} effect was time dependent. Thus, by pretreating with 10 μM Ca^{2+} for up to 120 min, a sevenfold increase of V_{max} was found while K_s was almost halved (Fig. 2). The effect seemed to be maximal after 60–90 min preincubation. Under these conditions, the enzyme behaved like a CaM-stimulated ATPase, as judged by the V_{max} and K_s values attained. Most of the experiments described below were restricted to 30 min pretreatment, in spite of a steady effect not being completely reached.

The Ca^{2+} activation was not abolished by omitting DTE from preincubation medium (Table 3) or adding a wide variety of proteinase inhibitors. Thus, previous incubation with Ca^{2+} plus either iodoacetamide (10 mM), leupeptin (200 μM), pepstatin A (100 μM) or PMSF (100 μM) only resulted in a slight inhibitory action (Table 2). Preliminary incubation with neomycin (200 μM) also had a negligible action.

The above stimulation was also minimally altered

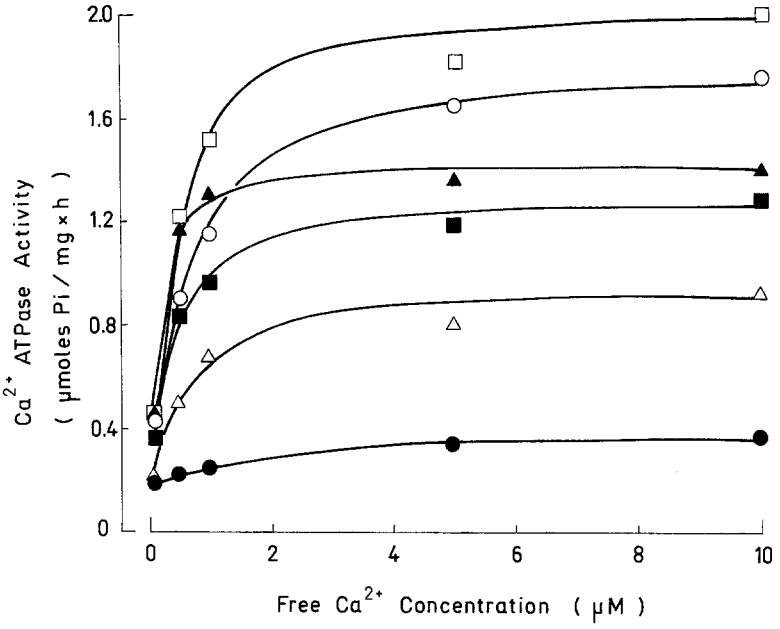

Fig. 1. The effect of pretreatment on Ca^{2+}-ATPase activity. CaM-free membrane fragments prepared from fresh human blood, were preincubated for 30 min at 37°C in a basal medium containing (mM): KCl, 130; ouabain, 0.1; EGTA, 1; tris-HCl (pH 7.55 at room temperature) 20; without (filled circles) or with the following additions: 10 mM $MgCl_2$ (open triangles), 10 µM free Ca^{2+} (filled squares), 10 mM $MgCl_2$ plus 10 µM free Ca^{2+} (filled triangles), 10 µM free Ca^{2+} plus 2 mM ATP (open circles) and 10 mM $MgCl_2$ plus 10 µM free Ca^{2+} plus 2 mM ATP (open squares). Thereafter, fragments were washed and incubated with 2 mM ATPMg for 2 hr at 37°C, in the basal medium described above but containing the various free Ca^{2+} concentrations shown in the graph. The results shown are mean values from 12 experiments run in duplicate. The SD was always less than 0.01 and was omitted for clarity.

Table 1. Kinetic parameters of Ca^{2+}-ATPase

Pretreatment	V_{max} (µmol P_i/mg × hr)	P	K_s (µM)	P
None	0.32 ± 0.002		0.49 ± 0.098	
None (CaM added)	2.04 ± 0.007	<0.005	0.15 ± 0.049	<0.005
After 30 min with:				
EGTA (1 mM)	0.38 ± 0.008	>0.01	0.30 ± 0.076	<0.01
$MgCl_2$ (10 mM)	0.95 ± 0.033	<0.005	0.45 ± 0.068	>0.01
Ca^{2+} (10 µM)	1.31 ± 0.023	<0.005	0.33 ± 0.058	<0.01
Ca^{2+} (10 µM) + $MgCl_2$ (10 mM)	1.43 ± 0.011	<0.005	0.15 ± 0.022	<0.005
Ca^{2+} (10 µM) + ATP (2 mM)	1.84 ± 0.032	<0.005	0.48 ± 0.057	>0.01
Ca^{2+} (10 µM) + ATP (2 mM) + $MgCl_2$ (10 mM)	2.05 ± 0.041	<0.005	0.38 ± 0.076	>0.01

V_{max} and K_s were calculated from linear regression analyses on Eadie-Hofstee plots of the experiments presented in Fig. 1. r^2 values were always greater than 0.996.

by extensive washing with EGTA after preincubation. Thus, after two washes with 5 mM EGTA, the relative activity (as percentage of control) of fragments pretreated with 10 µM Ca^{2+}, was 100 ± 3.8 and 96 ± 3.5 (mean ± 1 SD of four experiments) when assessed at 0.1 and 10 µM Ca^{2+}, respectively. Similarly, the extent of activation was reduced slightly when incubating and washing with delipidized bovine serum albumin (1 mg/ml). Under these conditions, the relative activity (as percentage of control) was 105 and 95 (mean of two experiments) when determined at 0.1 and 10 µM Ca^{2+}, respectively.

Addition of ATP during preincubation with Ca^{2+} further increased V_{max} without affecting K_s. The maximal effect was obtained with 1–2 mM ATP. However, lower concentrations also were activatory. Thus, the Ca^{2+}-ATPase activity assayed at 5 µM Ca^{2+} (average from two experiments) was 0.35, 0.54, 0.58, 1.01 and 1.15 µmol P_i/mg × hr after preincubating with no ATP or 0.01, 0.1, 1 and 2 mM ATP, respectively. An ATP-regenerating system consisting of 10 units creatine kinase/ml and 10 mM phosphocreatine was included in experiments testing low nucleotide levels.

The ATP effect was potentiated by the presence of

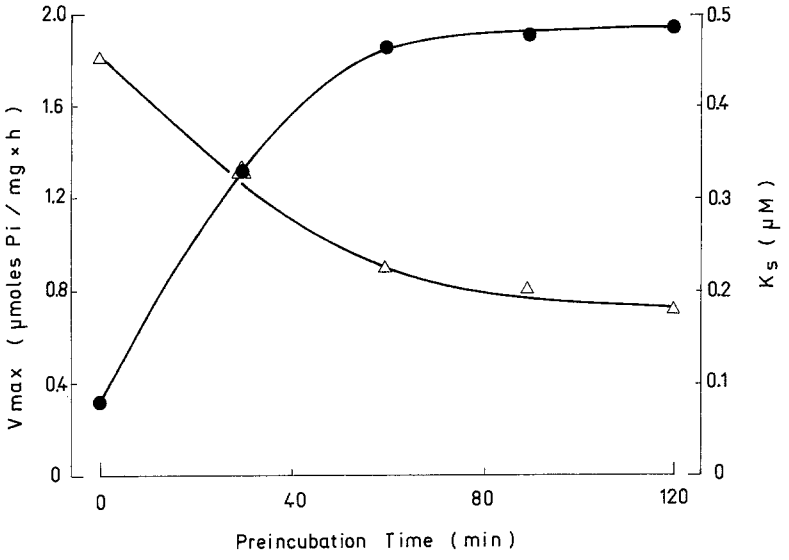

Fig. 2. Time course of Ca^{2+}-ATPase modification. CaM-free membrane fragments were preincubated with 10 µM free Ca^{2+} for the various times shown above and treated as described in the legend to Fig. 1. The enzyme V_{max} (filled circles) and K_s (open triangles) were calculated from linear regression analyses on Eadie-Hofstee plots. Results are the mean value of two experiments run in duplicate.

Table 2. Lack of effect of some agents during pretreatment

Conditions	Free Ca^{2+} concentration during ATPase assay		
	0.1 µM	10 µM	*n*
Control (10 µM Ca^{2+})	0.39 ± 0.0078	1.28 ± 0.008	8
Leupeptin (100 µM)	0.36 ± 0.017	1.16 ± 0.01	4
Pepstatin (100 µM)	0.36	1.21	2
Iodoacetamide (10 mM)	0.30	1.26	2
PMSF (100 µM)	0.36	1.31	2
Neomycin (1.5 mM)	0.42 ± 0.006	1.26 ± 0.0051	3
Delipidized BSA (1 mg/ml)	0.44	1.19	2
EGTA washing	0.37 ± 0.0038	1.24 ± 0.0035	3
DTE omission	0.37 ± 0.007	1.29 ± 0.0047	3

CaM-free membrane fragments were preincubated as described in the legend to Fig. 1, under conditions specified above. The Ca^{2+}-ATPase activity (in µmol P_i/mg × hr) was determined at 0.1 and 10 µM Ca^{2+}. Results are given as mean values ± 1 SD of the mean or the average of the number of experiments (*n*).

Mg^{2+} (10 mM), thus reaching a V_{max} of about 2.1 µmol P_i/mg × hr (Table 1). The latter stimulation was reduced 25–30% by incorporating acridine orange (100 µM) or compound 48/80 (100 µM) but not DTNB (1 mM) (Table 3). A similar reduction was also obtained by preincubating with leupeptin (200 µM). Thus, under this condition, the activity (mean value of three experiments) was reduced by 21.6 and 35.9% when measured at 0.5 and 10 µM Ca^{2+}, respectively.

At optimal Ca^{2+}, Mg^{2+} and ATP concentrations (10 µM, 10 mM and 2 mM, respectively), the extent of stimulation attained was similar to that found with CaM (0.3 µM) (Table 1). On the other hand, addition of the latter after preincubating with Ca^{2+} stimulated V_{max} by 15–45%, depending on free Ca^{2+} concentrations (*results not shown*).

The steady-state fluorescence depolarization (*P*) of DPH and TMA-DPH incorporated into fragments was practically unaltered by previous incubation with Ca^{2+} or Mg^{2+}. Thus, *P* for DPH was about 0.28 ± 0.002 (mean ± 1 SD of six experiments) for control membranes (incubated with 5 mM EGTA), a value not statistically different ($P > 0.1$) from that obtained after preincubation with Mg^{2+} or Ca^{2+}. Similarly, *P* for TMA-DPH was about 0.36 ± 0.006 (mean ± 1 SD of four experiments) for control membranes, not statistically different ($P > 0.1$) from the value obtained with Mg^{2+} or Ca^{2+}. The *P* values for both DPH and TMA-

Table 3. Effect of protein kinase inhibitors

Conditions of pretreatment	Activity at 0.5 μM Ca^{2+}	Relative inhibition (%)
Ca^{2+} (10 μM) + $MgCl_2$ (10 mM) + ATP (2 mM)	1.24	0
As above plus:		
Compound 48/80 (100 μg/ml)	0.94	24
DTNB (1 mM)	1.16	7
Acridine orange (100 μg/ml)	0.86	31

CaM-free membrane fragments were preincubated with various protein kinase inhibitors as described in the legend to Fig. 1. Ca^{2+}-ATPase activity (in μmol P_i/mg × hr) was determined at 0.5 μM Ca^{2+}. Results are given as mean values of five experiments, with a 1 SD value not greater than 0.12.

DPH of control membranes were decreased significantly ($P < 0.005$) when 10–20% (vol/vol) ethanol was added to the measuring cuvette, showing that the probes were sensing changes in membrane fluidity.

SDS-PAGE gels revealed no differences in the electrophoretic pattern of membrane polypeptides after preincubating for 1 hr at 37°C with EGTA (5 mM), Ca^{2+} (10 μM) or Mg^{2+} (10 mM) plus Ca^{2+} (10 μM) (Fig. 3). Similarly, immunodetection of Ca^{2+}-ATPase did not reveal any alteration in enzyme mobility under above conditions (*results not shown*).

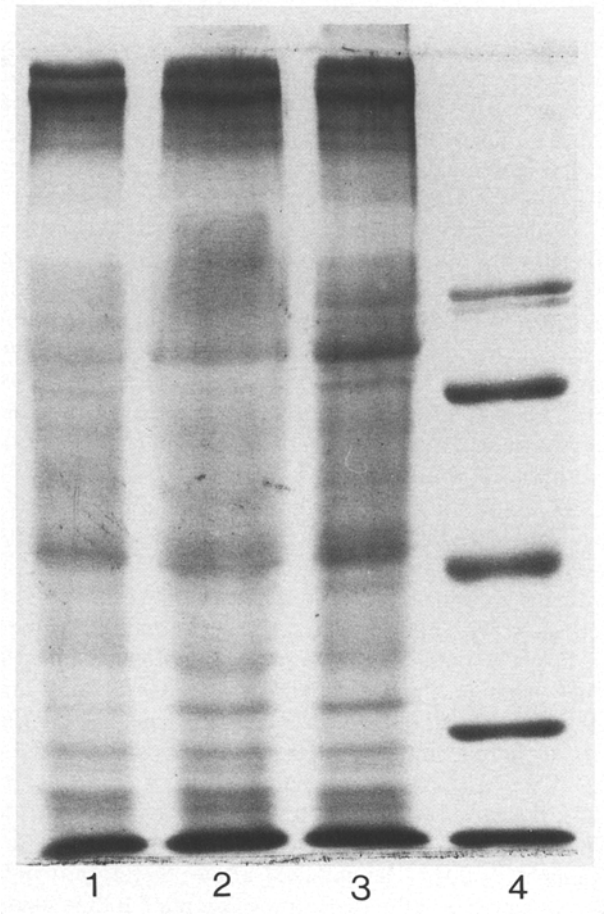

Fig. 3. SDS-PAGE gel of CaM-free membrane fragments after various treatments. The lanes (from left to right) show membranes pretreated for 1 hr at 37°C without divalent cations (*1*) or with 10 mM $MgCl_2$ (*2*) or 10 μM free Ca^{2+} (*3*) (1 mM EGTA being present throughout). Lane *4* (from top to bottom) shows low molecular weight markers (MW): bovine serum albumin (66,000); fumarase (48,500); carbonic anhydrase (29,000); β-lactoglobulin (18,400) and lactalbumin (14,200).

Discussion

The present work has shown that the human erythrocyte Ca^{2+}-ATPase is markedly stimulated after preincubation with micromolar Ca^{2+} levels. Under this condition, both V_{max} and Ca^{2+} affinity are increased. These results are in agreement with early work, showing that preincubation of the red cell Ca^{2+}-ATPase with Ca^{2+} (at millimolar levels) results in enzyme activation [2, 15, 30].

As in the latter case [30], the effect found in this work was also obtained in the complete absence of reducing agents or in the presence of a wide variety of proteinase inhibitors [8], thereby eliminating the possibility of being mediated by proteolysis. Furthermore, densitometric analyses of membrane polypeptides revealed no change in electrophoretic pattern after preincubating with or without Mg^{2+}, Ca^{2+} or Mg^{2+} plus Ca^{2+}. In addition, immunodetection of Ca^{2+}-ATPase did not reveal any alteration in enzyme mobility after pretreatment.

Activation by Ca^{2+} is associated to neither phosphatidylinositol phosphate metabolism nor lipid release, as inferred from the following considerations. First, it is known that Ca^{2+} catalyzes hydrolysis of phosphatidylinositol phosphates [22], well-known activators of plasma membrane Ca^{2+}-ATPases [10]. Secondly, Ca^{2+} has also been shown to stimulate phospholipase A_2 activity, thus leading to fatty acids and glycerol, which in turn, may stimulate the Ca^{2+}-ATPase [5, 36]. However, both incubating and washing with delipidized albumin did not abolish the Ca^{2+} action, showing that it is not related to fatty acid release. Finally, incubation with neomycin, a specific phospholipase C inhibitor, is also without effect.

It is well known that Ca^{2+} interacts with acidic phospholipids inducing phase transitions and lateral segregation of lipid domains [14]. Thus, the possibility exists that an increased membrane fluidity arising as consequence of Ca^{2+} pretreatment can mediate its action. In this work, however, it was shown that the flu-

orescence depolarization of DPH and TMA-DPH, used here as probes for changes in membrane viscosity [34], was not affected by previous incubation with Ca^{2+} or Mg^{2+}. These results clearly demonstrate that no detectable changes in membrane fluidity arise from pretreatment with the above modulators.

The above observations suggest that a ligand-induced conformational change is probably involved in Ca^{2+} activation, as has been already suggested by others [30]. This idea receives additional support from the work of Bond [7], who reported changes in proteolytic, thermal and sulfhydryl reagent susceptibilities of the human red cell Ca^{2+}-ATPase after preincubating with either Ca^{2+} or Mg^{2+}. As the Ca^{2+}-ATPase activation was found in the presence of both Ca^{2+} and EGTA, a condition known to stabilize or promote the E_1 conformation [1], the latter conformer is likely to be involved.

Perhaps the most important finding was that preincubation with micromolar Ca^{2+} levels induced a kinetic behavior both qualitative and quantitatively similar to that promoted by CaM, increasing Ca^{2+} affinity and V_{max}. It is known that CaM raises V_{max} and Ca^{2+} affinity, by interacting with different domains on the ATPase [9, 11]. As CaM has no effect on ATP-P_i exchange [6, 25], the raised turnover rate is kinetically attributed to an increase in both phospho- and dephosphorylation rates [1, 4, 24]. The net CaM effect, therefore, would be an increase in the E_2-E_1 transition rate. It thus seems possible that the same conformational change attained after CaM binding (a hypothetical active state of the ATPase molecule, leading to an increased E_2 to E_1 transition rate) or the stabilization of the E_1 conformer as suggested for CaM action [1], is reached via preliminary incubation with Ca^{2+}.

In view of the fact that the activatory action of Ca^{2+} (or Mg^{2+}, *see below*) was not overcome by washing with chelating agents, it seems possible that either Ca^{2+} (or Mg^{2+}) became occluded or the new conformer attained upon ion binding remained stable after releasing the modulating ions.

This work also showed that preincubation with Mg^{2+} leads to enzyme activation, but contrary to previous work [27], the effect is achieved in the absence of vanadate. This difference certainly arises from the reduced basal Ca^{2+}-ATPase activity of control fragments attained in the present work. Thus, a V_{max} of about 0.4 μmol P_i/mg × hr is obtained in nonincubated fragments or after preincubating for 30 min in the presence of EGTA (1 mM). By contrast, in a previous work, a V_{max} of about 1 μmol P_i/mg × hr was obtained as control activity after preincubating for 60 min with 5 mM EGTA [27]. We do not know the reasons for this discrepancy. However, it has been reported that under certain conditions, presence of EGTA elicits an increased Ca^{2+}-ATPase activity in human red cell membranes [3, 29]. Perhaps the above discrepancies in basal Ca^{2+}-ATPase activity may arise from a shorter exposure to lower EGTA concentrations as we have presently used.

A final point to be addressed is the effect of ATP. Addition of the nucleotide during Ca^{2+} preincubation with or without Mg^{2+}, increased V_{max} by about 25–40% without affecting K_s. The stimulation obtained in the presence of Mg^{2+} was reduced around 25–30% by including compound 48/80 or acridine orange, known inhibitors of Ca^{2+}/CaM protein kinase and PKC, respectively (for references, *see* [28]). These findings suggest that the above kinases are involved and that ATP is being metabolized during preincubation. It is known that Ca^{2+} pump phosphorylation by PKC leads to an increase in V_{max} but not K_s [35], an effect similar to that presently found with ATP. Moreover, this work showed that leupeptin reduced ATP stimulation by about 30%. Such a finding is consistent with a calpain-mediated effect on PKC during preincubation, as has been proposed recently [23].

The above observations are not in accordance with an early work [30], showing enzyme activation by nonmetabolizable ATP analogues. On the other hand, the lack of effect of DTNB, a protein kinase A inhibitor, makes unlikely an involvement of this kinase.

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