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Synergistic activation of the human red cell calcium ATPase by magnesium and vanadate

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The Ca^{2+}-ATPase activity of human red cells was studied on calmodulin-free membrane fragments after previous incubation with Mg^{2+} and vanadate. In the presence of EGTA (5 mM), the activity was slightly affected by either ion alone. However, when added together, both Ca^{2+} affinity and V_{max} were increased up to levels found with calmodulin (0.3 μM). This synergistic activation was not abolished by proteinase inhibitors (iodoacetamide, 10 mM; leupeptin, 200 μM; pepstatin A, 100 μM; phenylmethanesulfonyl fluoride, 100 μM), neomycin (200 μM), washing with EDTA (5 mM) or by both incubating and washing with delipidized serum albumin (1 mg/ml). During preincubation under optimal Mg^{2+} and vanadate conditions, the replacement of K^+ by Na^+ or Li^+ was without effect. Co^{2+} or Zn^{2+} (10 mM) could not substitute for Mg^{2+}, whereas Mn^{2+} almost replaced it at equimolar amounts. By contrast, addition of ATPMg (2 mM) decreased the activation by about one-half. Like calmodulin, pretreatment with Mg^{2+} plus vanadate also increased the affinity for ATP and elicited appearance of a second (low) affinity site (apparent $K_m = 120$ μM). 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 with Mg^{2+}, vanadate or Mg^{2+} plus vanadate. The results show that Mg^{2+} and vanadate are acting neither via proteolysis or fatty acid production nor by facilitating phospholipid metabolism or altering membrane fluidity. They may be enhancing the Ca^{2+}-ATPase activity by stabilizing the E_1 conformer or promoting an enzyme conformation which facilitates the E_2–E_1 transition.

Introduction

The human red cell Ca^{2+}-ATPase is activated by several effectors or conditions that may occur physiologically, such as CaM, acidic phospholipids, unsaturated fatty acids and proteolysis [1], autoaggregation [2] or phosphorylation by some protein kinases [3,4].

The enzyme alternates between the E_1 and E_2 conformations during its catalytic cycle [5–8]. The E_1 conformer having a high Ca^{2+} affinity can be phosphorylated by ATP, whereas the E_2 one, possessing a low Ca^{2+} affinity, is capable of being phosphorylated by P_i but not ATP [9,10]. It has been further shown that these conformers can be selectively sequestered by either La^{3+} (E_1) or vanadate (E_2) [8,11].

On the other hand, it was reported earlier that the above enzyme underwent conformational changes when previously incubated with Ca^{2+} or Mg^{2+} [12]. As a similar Ca^{2+} treatment results in an increased ATPase activity of human erythrocyte membranes [13], it was of interest to study on the same system the effect of previous incubation with Mg^{2+}. Here it is shown that, only in the presence of vanadate, Mg^{2+} stimulates the enzymic activity up to levels found with CaM.

Materials and Methods

All reagents were of analytical quality whenever possible, obtained mainly from Sigma, USA, and The British Drug Houses, UK.

Fresh O(+) blood from healthy human donors was used. CaM-free membrane fragments were prepared as described elsewhere [14] and stored at −80°C until use within not more than 30 days.

Preincubation of fragments was done for 60 min at 37°C under covered conditions in a water-bath

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Abbreviations: CaM, calmodulin; EGTA, (ethylenebis(oxyethylenenitrilo))tetraacetic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium phenyl)-6-phenyl 1,3,5-hexatriene; PI, phosphatidylinositol; PIP_2, phosphatidylinositol bisphosphate; PMSF, phenylmethanesulfonyl fluoride; K_s, apparent stability constant.

equipped with a lid, in the presence of 150 mM KCl, 5 mM EGTA and 100 mM Tris-HCl (pH 7.55 at room temperature), with and without Na_3VO_4 plus the additions specified below. Thereafter, the fragments were washed twice with an ice-cold 150 mM KCl + 10 mM Tris-HCl (pH 7.55) medium, containing 50 mM P_i to facilitate vanadate removal. Two further washes were done without P_i and fragments were finally resuspended in a small volume of washing medium to obtain about 10 mg protein/ml.

ATPase assays. Ca^{2+}-ATPase activity of previously incubated membranes was determined after 2 h 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 μM and 1 mM. The required amounts of Ca^{2+} and EGTA were calculated using a computer based program [15]. Ca^{2+}-ATPase activity was obtained by subtracting the activity found in the presence of Ca^{2+} from that in its absence. Ca^{2+}-ATPase activity was linear with time for up to 2 h incubation.

Membrane lipid fluidity Fluidity was assessed by monitoring the steady-state fluorescence depolarization of DPH and TMA-DPH [16]. These were incorporated by preincubating membrane fragments as described above in the presence of 100 μM of either fluorophore. A SLM polarization fluorimeter (Champaign, IL, USA) was used with a cell compartment held at 37 ± 0.5°C by an external thermostated water-bath. Measurements were corrected for light scattering effects [17], by determining the fluorescence depolarization at different dilutions of membrane suspension (absorbance range 0.03–050 at 450 nm). These corrections never exceeded 5%. An excitation wavelength of 359 nm was employed.

SDS–polyacrylamide gel electrophoresis. Membrane fragments were preincubated as above either in the dark (samples covered with aluminium foil) or under common room-light conditions. Thereafter, they 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 BioRad video densitometer (model 620).

P_i determinations. Determination of P_i were done by a modification of the Fiske and Subbarow method [19], using $FeSO_4$ as reducing agent.

Proteins. Proteins were determined by the Lowry method [20], using bovine serum albumin as standard.

Analyses of statistical significance. Statistical analyses were done using Student's *t*-tests.

Results

The Ca^{2+}-ATPase activity was slightly affected by previous incubation with either Mg^{2+} (2–10 mM) or vanadate (50–150 μM). Under these conditions, the enzyme appeared in a low-Ca^{2+} affinity (K_s about 0.2 μM) and low V_{max} (1.0–15 μmol P_i/mg protein per h) state (Fig. 1). However, when vanadate (50 μM) and Mg^{2+} (10 mM) were added together, both Ca^{2+} affinity and V_{max} were increased (K_s about 0.05 μM and 2.5 μmol P_i/mg protein per h, respectively) (Fig. 1). At a constant vanadate concentration (50 μM), increasing Mg^{2+} from zero up to 10 mM resulted in a progressive increase of ATPase activity, leveling off at about 5–10 mM (Fig. 2a). Conversely, at 10 mM Mg^{2+}, raising vanadate from 0 up to 150 μM also increased ATPase activity monotonically, apparently reaching a plateau between 50 and 100 μM (Fig. 2b).

At optimal vanadate and Mg^{2+} concentrations (50 μM and 10 mM, respectively), the extent of stimulation reached was similar to that found with CaM (0.3 μM). Moreover, addition of the latter was without effect after preincubation with both Mg^{2+} and vanadate, whilst it stimulated by 100–500% (depending on free Ca^{2+} concentration) when fragments were preincubated with or without either ion alone (Table I).

The Mg^{2+} plus vanadate activation was not abolished by presence of a wide variety of proteinase inhibitors. Thus, previous incubation with the modulators plus either iodoacetamide (10 mM), leupeptin (200 μM), pepstatin A (100 μM) or PMSF (100 μM) only resulted in a slight inhibitory action. The most potent effect was found with the latter inhibitor, being only about 20%. Similarly, preliminary incubation with either fluoride (5 mM) or neomycin (200 μM) had negligible action.

The above stimulation was also minimally altered by extensive washing with EDTA after preincubation.

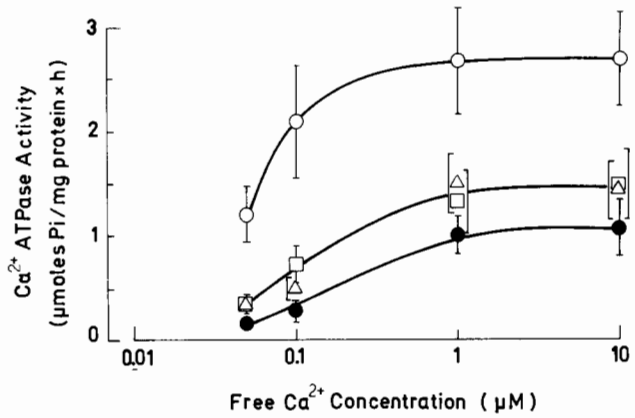

Fig. 1. Synergistic activation of Ca^{2+}-ATPase by Mg^{2+} plus vanadate. Fragments were preincubated for 1 h at 37°C in a medium containing (mM): 150 KCl+5 EGTA+100 Tris-HCl (pH 7.55 at room temperature), with no additions (●) or in the presence of 10 mM $MgCl_2$ (△), 50 μM Na_3VO_4 (□) and 10 mM $MgCl_2$ plus 50 μM Na_3VO_4 (○). After extensive washing, Ca^{2+}-ATPase activity of these fragments was assayed by incubating for 2 h at 37°C in the presence of the various free Ca^{2+} concentrations shown above. Results are given as mean values ± 1 S.D. of 7 experiments.

TABLE I

Effect of CaM after preincubation with and without Mg^{2+} and vanadate

Fragments were preincubated as described in legend to Fig. 1 with the additions indicated above. After washing, Ca^{2+}ATPase was assayed in the presence and absence of CaM (0.3 μM). The results are given as mean values ± 1 S.D. of seven experiments.

Preincubation conditions	Stimulation by CaM (%) with	
	0.1 μM Ca^{2+}	10 μM Ca^{2+}
Control	568 ± 377.0	106 ± 24.3
50 μM VO_4^{3-}	380 ± 294.3	91 ± 20.4
10 mM Mg^{2+}	288 ± 82.6	113 ± 34.9
Mg^{2+} + VO_4^{3-}	2 ± 4.7	12 ± 7.1

TABLE II

Effect of divalent cations during preincubation with vanadate

Fragments were preincubated as described in legend to Fig. 1, in the presence of 50 μM vanadate and additions as shown above. Then, they were washed five times with a 150 mM KCl + 10 mM Tris-HCl (pH 7.4) solution, containing 1 mM EDTA, before assaying Ca^{2+}ATPase activity. Results from 3 experiments are presented.

Added cation (10 mM)	Ca^{2+}-ATPase activity (μmol P_i /mg protein/h) with:			
	0.1 μM Ca^{2+}		10 μM Ca^{2+}	
	− CaM	+ CaM	− CaM	+ CaM
None	0.51	2.40	1.52	2.82
$MgCl_2$	1.35	2.40	2.09	2.65
$MnCl_2$	1.31	1.80	0.96	1.29
$CoCl_2$	0.19	0.20	0.29	0.45
$ZnSO_4$	0.07	0.14	0.06	0.12

Thus, after 5 washes with 5 mM EDTA, the relative activity (as % of control) of fragments that were preincubated with 50 μM vanadate plus 10 mM Mg^{2+}, was 91 ± 6.4 and 89 ± 5.8 (mean ± 1 S.D. of 4 experiments) when assessed at 0.1 and 10 μM Ca^{2+}, respectively. Similarly, the extent of activation was reduced slightly by both incubating and washing with delipidized bovine serum albumin (1 mg/ml). Under these conditions, the relative activity (as% of control) was 73 ± 8.5 and 79 ± 11.4 (mean ± 1 S.D. of 4 experiments) when assessed at 0.1 and 10 μM Ca^{2+}, respectively.

In the presence of vanadate (100 μM), Co^{2+} or Zn^{2+} (10 mM) could not substitute for Mg^{2+} whereas equimolar Mn^{2+} concentrations almost replaced it, producing the same stimulation found with Mg^{2+} at low Ca^{2+} (0.1 μM) and about a half of that obtained at saturating Ca^{2+} (Table II). The replacement of K^+ by Na^+ or Li^+ as main monovalent cation was without effect (results not shown). By contrast, addition of ATPMg (2 mM) during preincubation almost prevented Mg^{2+} plus vanadate action. Accordingly, ATPase activity was increased only 2-fold above control values when ATPMg was added instead of being raised about 8-times after preincubating without ATP (Table III). On the other hand, ATP addition to activated fragments slightly reduced the activatory effect of Mg^{2+} plus vanadate on a second preincubation. Conversely, further treatment with vanadate plus Mg^{2+} of fragments previously incubated with these ions in the presence of ATP, resulted in partial stimulation of the ATPase activity. These results show that ATP does not reverse Mg^{2+} plus vanadate action once it has been established.

In addition to the effect described above on Ca^{2+} affinity and V_{max} previous incubation with vanadate and Mg^{2+} also increased ATP affinity at the high affinity site and elicited the appearance of a low affinity site (Fig. 3). Thus, linear regression analyses of reciprocal plots showed a K_m for ATP of about 100 μM ($r^2 = 0.995$) after control preincubation. By con-

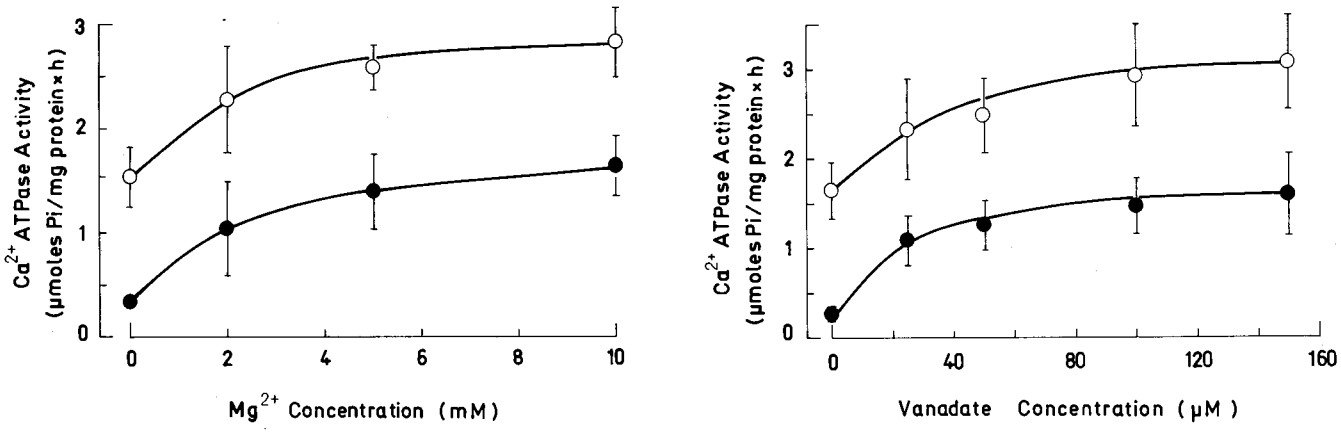

Fig. 2. Requirements for Mg^{2+} and vanadate. Fragments were treated as described in the legend to Fig. 1, but preincubating at a constant vanadate concentration (50 μM) and variable Mg^{2+} levels (a) or at constant Mg^{2+} (10 mM) and variable vanadate concentrations (b). Ca^{2+}-ATPase activity was assayed at 1 (●) and 10 μM free Ca^{2+} (○). Collected results from at least 4 experiments are presented in the graph as mean values ± 1 S.D.

TABLE III

The effect of ATP on the synergistic activation by Mg^{2+} plus vanadate

Fragments were preincubated as described in legend to Fig. 1, for 1 h at 37°C in the absence or presence of 10 mM $MgCl_2$ plus 150 μM vanadate, with (condition b) and without 2 mM ATP (condition a). After extensive washing, a portion of fragments from conditions a and b were further incubated for one more hour either with ATP (2 mM) or Mg^{2+} (10 mM) plus vanadate (150 μM), respectively. Results are the average value of 3 experiments.

Preincubating for 1 h at 37°C with	Ca^{2+}-ATPase activity (μmol P_i/mg protein per h) with:			
	0.1 μM Ca^{2+}		10 μM Ca^{2+}	
	−CaM	+CaM	−CaM	+CaM
0 Mg^{2+}/0 VO_4^{3-}	0.38	2.06	0.80	2.24
10 mM Mg^{2+} + 150 μM VO_4^{3-} (a)	2.24	2.32	2.54	2.51
10 mM Mg^{2+} + 150 μM VO_4^{3-} + 2 mM ATPMg (b)	0.99	1.98	1.45	2.56
After (a) plus 1 h more with 2 mM ATPMg	1.43	2.03	2.11	2.28
After (b) plus 1 h more with 10 mM Mg^{2+} + 150 μM VO_4^{3-}	1.44	2.07	1.75	2.22

trast, when fragments were previously incubated with Mg^{2+} plus vanadate there appear two sites, having K_m values of about 6 μM ($r^2 = 0.999$; range 0.5–50 μM ATP) and 212 μM ($r^2 = 0.986$; range 100–2000 μM ATP). This can be compared to the corresponding values of control membranes assayed in the presence of saturating CaM concentrations (0.3 μM), namely 3 μM ($r^2 = 0.984$; range 0.5–50 μM ATP) and 120 μM ($r^2 = 0.983$; range 100–2000 μM ATP).

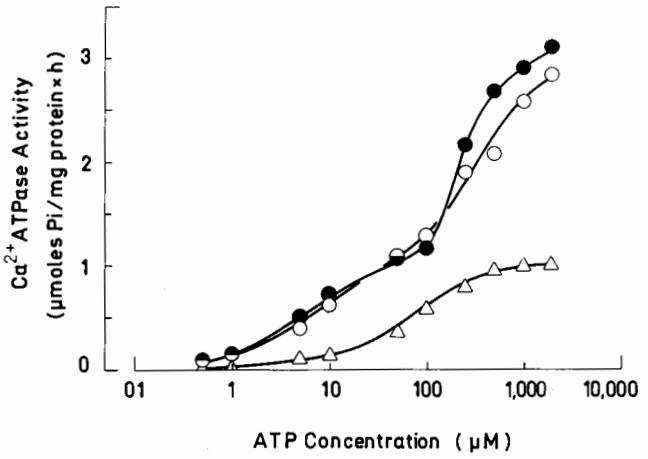

Fig. 3. Appearance of a low-affinity ATP-binding site after preincubation with Mg^{2+} plus vanadate. Fragments were preliminarily incubated in the absence and presence of 50 μM vanadate plus 10 mM $MgCl_2$, as described in the legend to Fig. 1. After washing, Ca^{2+}-ATPase activity was assessed at 10 μM free Ca^{2+} and the different ATP concentrations shown above, by incubating for 2–4 h at 37°C in the presence of 10 mM creatine phosphate and 10 units creatine phosphokinase/ml. The graph shows the activity after preincubating with (●) and without Mg^{2+} plus vanadate (○). ATPase activity of control fragments (△) was also assessed in the presence of 0.3 μM CaM (circles). Results shown are mean values of 3 experiments.

The steady-state fluorescence depolarization (P) of DPH and TMA-DPH incorporated into fragments was practically unaltered by previous incubation with and without vanadate and Mg^{2+}. Thus, P for DPH was about 0.28 ± 0.002 (mean ± 1 S.D. of 6 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+} plus vanadate. Similarly, P for TMA-DPH was about 0.36 ± 0.006 (mean ± 1 S.D. of 4 experiments) for control membranes, not statistically different ($P > 0.1$) from the value obtained with Mg^{2+} plus vanadate. The P-values for both DPH and TMA-DPH of control membranes were decreased in a statistically significant way ($P < 0.005$) when 10–20% (v/v) ethanol was added to the measuring cuvette. This shows that the probes were sensing changes in membrane fluidity.

Densitometric analyses of SDS–PAGE gels revealed no differences in the electrophoretic pattern of membrane polypeptides after preincubating for 1 h at 37°C with EGTA (5 mM), Mg^{2+} (10 mM), vanadate (50 μM) or Mg^{2+} (10 mM) plus vanadate (50 μM), either in the dark or under illumination (results not shown).

Discussion

The present work has shown a marked stimulation of the human erythrocyte Ca^{2+}-ATPase after preliminary incubation with Mg^{2+} plus vanadate. Under this condition, both V_{max} and Ca^{2+} affinity were increased. As the activating ions have no appreciable effect on their own, their effect is truly synergistic. Such an activation was found in the complete absence of both Ca^{2+} and reducing agents or in the presence of a wide variety of proteinase inhibitors [21], thereby eliminating the possibility of being mediated by proteolysis.

The synergistic effect is associated neither to phosphatidylinositol phosphate metabolism nor lipid release, as inferred from the following considerations. First, it is known that vanadate activates incorporation of γ-phosphate from ATP into phosphatidylinositol phosphates [22], well known activators of plasma membrane Ca^{2+}-ATPases [23]. Nonetheless, ATP addition prevented enzyme activation by Mg^{2+} plus vanadate. Secondly, vanadate has also been shown to stimulate lipoprotein-lipase activity [24]. However, incubation and washing with delipidized albumin did not abolish the synergistic action, thus showing that it is not related to fatty acid release. Finally, presence of Li^+, a known inhibitor of inositol phosphate hydrolysis, or incubation with neomycin, a specific phospholipase C inhibitor, are also without effect.

It is also known that vanadate catalyses photocleavage of various proteins, including the sarcoplasmic

reticulum (SR) Ca^{2+}-ATPase [25]. This possibility is highly unlikely to have occurred in the present work, for the following reasons. First, Ca^{2+}-ATPase stimulation was observed only after preincubating with both vanadate and Mg^{2+}, not with vanadate alone. Secondly, in all published reports, ultraviolet light has been used as energy source to induce photocleavage of membrane proteins. By contrast, our experiments were done under nearly dark conditions. Finally, densitometric analyses of membrane polypeptides revealed no change in the electrophoretic pattern after preincubation with or without Mg^{2+}, vanadate or Mg^{2+} plus vanadate, either in the dark or under illumination.

On the other hand, the possibility exists that an increased membrane fluidity arising as consequence of pretreatment with Mg^{2+} plus vanadate can mediate their action. In this work, however, it was shown that the fluorescence depolarization of DPH and TMA-DPH, used here to probe changes in membrane viscosity [16], was not affected by previous incubation with Mg^{2+}, vanadate or Mg^{2+} plus vanadate. These results clearly demonstrate that no detectable changes in membrane fluidity arise from pretreatment with the above modulators.

It was shown recently that preliminary incubation of the red cell Ca^{2+}-ATPase with millimolar Ca^{2+}, also results in enzyme activation [13]. This effect could neither be accounted for by calpain activation nor by phospholipid or fatty acid release. According to the authors, the activation may be related to a Ca^{2+}-induced conformational change. This idea receives additional support from the work of Bond [12], 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+}.

The above observations suggest that a Mg^{2+}-induced conformational change is probably involved in the synergistic activation found in the present work As the effect was seen only in the presence of both EGTA and vanadate, conditions known to stabilize the E_2 conformation, the latter conformer is likely to be involved. It is important to stress that vanadate is a powerful Ca^{2+}-ATPase inhibitor [26,27], and must be released first from the E_2 conformer for restoring ATPase activity. Since the synergistic action was not overcome by washing with EDTA, it seems possible that either Mg^{2+} became occluded, or the new conformer attained upon Mg^{2+} binding remained stable after releasing the modulating ligands.

By analogy with the (SR) Ca^{2+}-ATPase (for review see Ref. 28), the rate-limiting step of the red cell enzyme may be the transition E_2 to E_1. This step has been postulated to be favoured by ATPMg, the putative low-affinity site [5]. In this work, it was shown that ATP prevented activation by both vanadate and Mg^{2+} but remained ineffective once it was developed. With the SR enzyme there is evidence that the complex ATPMg–vanadate interacts with the E_2 conformer, stabilizing it [29]. Moreover, vanadate binds at two different sites with high (micromolar) and low (milimolar) affinity [30]. This may suggest interaction with both low and high affinity ATP binding sites. If the above statement also applies to the red cell, the complex ATPMg–vanadate may induce a different conformation to that elicited by Mg–vanadate. As with the SR enzyme, it may also be possible that vanadate is released from the red cell enzyme more slowly from the ATPMg–vanadate complex than from the Mg–vanadate complex. Under such a condition, the enzyme would be partially locked at the E_2 conformation and the ATPase activity consequently reduced.

Perhaps the most important finding was that previous incubation with vanadate plus Mg^{2+} induced a kinetic behaviour both qualitatively and quantitatively similar to that promoted by CaM. In addition to increasing Ca^{2+} affinity and V_{max}, a second site for ATP was also elicited under this condition. It is now known that CaM increases $V_{max.}$ and affinity for both Ca and ATP, by interacting with different domains on the ATPase [31,32]. As CaM does not affect the extent of the ATP-P_i exchange reaction [33,34], the raised turnover rate achieved with CaM is kinetically attributed to an increase in both phospho- and dephosphorylation rates [35,36]; the net effect, therefore, being an increase in the E_2–E_1 transition rate, the putative rate-limiting step. 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–E_1 transition rate) or the stabilization of the E_1 conformer, is reached via preliminary incubation with Mg^{2+} plus vanadate. This suggestion may be extended to the purified enzyme treated with organic solvents [33].

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