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Rapid Report

Effects of magnesium plus vanadate on partial reactions of the Ca^{2+}-ATPase from human red cell membranes

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Abstract

Under conditions in which pretreatment with Mg^{2+} plus vanadate activate the Ca^{2+}-ATPase, the initial rate of phosphorylation of the enzyme increased from 141 to 259 pmol/mg protein per s while the steady-state level of phosphoenzyme lowered from 1.9 to 1.1 pmol/mg protein. The drop in phosphoenzyme level was caused by incubation and washing during treatment rather than by vanadate. The data allowed to estimate a turnover number for the enzyme that raised by 170% after pretreatment. The results show that the activation of the Ca^{2+}-ATPase by Mg^{2+} plus vanadate is due to changes in the kinetic properties of the enzyme.

Keywords: ATPase, Ca^{2+}-; Phosphorylation; Vanadate-activated phosphorylation; Calcium-ion-dependent phosphoenzyme; Phosphoenzyme

It has been simultaneously shown by Bond and Hudgins [1] and by us [2] that in media with Mg^{2+} and K^+, vanadate (VO_3^-) inhibits with high affinity ($K_i = 3$ μM) the Ca^{2+}-ATPase activity of human red cell membranes. Inhibition is associated with a parallel decrease in the steady-state level of the Ca^{2+}-dependent phosphoenzyme (EP) [2]. We have shown recently that, in sharp contrast with these findings, calmodulin-free membranes that have been incubated with vanadate in media with Mg^{2+} and K^+ increase their Ca^{2+}-ATPase activity after extensive washing with KCl and Na_2HPO_4 [3]. Activation persists after washing the membranes with EDTA and is not mediated by proteolysis, fatty acids, phospholipids nor changes in membrane fluidity [3]. Like calmodulin, pretreatment with Mg^{2+} plus vanadate followed by washing, increases the apparent affinity of the Ca^{2+}-ATPase for Ca^{2+} and elicits the appearance of the second (low affinity) site for ATP [3].

These results raised the question on whether vanadate and washing increases the Ca^{2+}-ATPase activity of red cell membranes either by changing the kinetic properties of the enzyme or by disclosing Ca^{2+}-ATPase units otherwise silent in the membranes. To solve this question, the time course of phosphoenzyme formation of the Ca^{2+}-ATPase from intact human red cell membranes [4] and from the same membranes treated with Mg^{2+} plus vanadate [3], was measured at 37° C using a chemical quenching technique that allows pre-steady-state measurements [5]. Phosphorylation curves were fitted by non-regression analyses using a GPAD INPLOT software.

Results in Fig. 1 show that in control membranes, the concentration of EP raised along a Michaelis-Menten like curve that reached a steady value of about 1.9 pmol/mg protein after 40 ms. In membranes pretreated with Mg^{2+} plus vanadate, EP also raised along a Michaelis-Menten like curve that reached a steady value of 1.1 pmol/mg protein. Most of the experimental points on the initial part of the curve corresponding to the membranes that had been pretreated with vanadate fall on the left side of the control curve, making clear that after treatment, phosphorylation of the Ca^{2+}-ATPase was faster. Apparent first order rate constant (k_{app}) values of 75 s^{-1} for the control and 244 s^{-1} for the treated enzyme were obtained from regression analyses. The initial rate of phosphorylation was estimated to be 141 and 259 pmol/mg protein per s in control and treated membranes, respectively.

A lower level of EP concentration at steady-state in Fig. 1 was taken as indicative that treatment of the membranes with Mg^{2+} plus vanadate followed by washing either raised the proportion of unphosphorylated forms of the Ca^{2+}-ATPase during the reaction cycle or inactivated a

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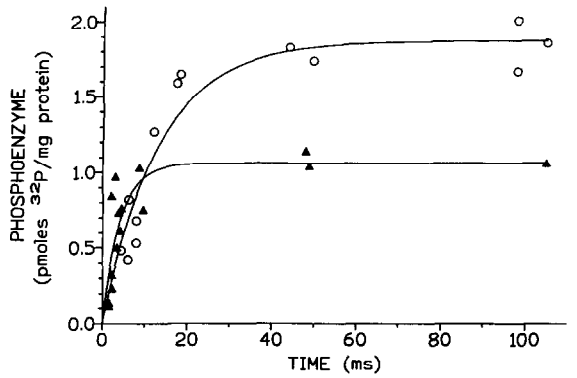

Fig. 1. Time-course of phosphorylation of the Ca^{2+}-ATPase before (○) and after (▲) treatment with Mg^{2+} plus vanadate. Human red cell membranes freed of calmodulin were prepared as described before [4] and kept at −80° C until used within no more than 5 days. After thawing, one portion of the membrane suspension was kept as the control and the other was preincubated for 1 h at 37° C in a medium containing (mM): KCl, 150; EGTA-Tris, 5; $MgCl_2$, 10; NH_4VO_3, 0,15; Tris-HCl (pH 7.55 at room temperature), 100. Thereafter, the membranes were washed first with an ice-cold 150 mM KCl and 50 mM Na_2HPO_4 solution, then with a 150 mM KCl solution and finally with a 100 mM choline-HCl and 50 mM Tris-HCl solution and resuspended in more of the last solution. Control and pretreated membranes were incubated in the phosphorylation media without ATP at 37° C during 30 min and then phosphorylated as before [5] in 100 mM choline-HCl; 50 mM Tris-HCl; 20 μM [γ-^{32}P]ATP (specific activity 500–1500 cpm/μmol), 0.5 mM EGTA; 2 mM $MgCl_2$, with and without 0.6 mM $CaCl_2$. EP was the difference between the amount of ^{32}P incorporated into the membrane protein in this medium and in medium of identical composition except that $CaCl_2$ was omitted. Collected results from three experiments are shown as the average value of 3–5 replicates.

fraction of the enzyme units originally present in the membranes. To test this point, the concentration of EP was measured in control membranes and in membranes that were submitted to incubation and washing as those treated with Mg^{2+} plus vanadate except that vanadate was omitted from all media. After phosphorylation during 5 s at 37° C, EP was 1.52 and 1.01 pmol P_i/mg protein in control and in incubated membranes, respectively, showing that incubation without vanadate followed by washing lowered to 66% of the control the amount of EP at steady-state, that is just about the drop in EP level observed in Fig. 1. This result was confirmed in two additional experiments. It seems therefore that the lower level of EP at steady-state shown in Fig. 1 could had been due to partial inactivation of the enzyme caused by the incubation and washing of the membranes during the treatment rather than by vanadate itself. After this, it seemed reasonable to think that the increase in phosphorylation rate observed in Mg^{2+} plus vanadate-treated membranes (Fig. 1) could also be due to incubation and washing of the membranes rather than to vanadate. Nevertheless, previous results (Fig. 3 in [3]) showing that, unless vanadate is present, preincubation and washing of the membranes does not change the shape of the activity vs. ATP concentration curve of the Ca^{2+}-ATPase, rule out that possibility.

The effects of treatment of the membranes with Mg^{2+} plus vanadate on phosphorylation are shown in Table 1 together with the Ca^{2+}-ATPase activity measured in media of composition similar to those used during phosphorylation. Under these conditions, pretreatment with Mg^{2+} plus vanadate increased from 62.2 to 95.6 pmol/mg protein per s the Ca^{2+}-ATPase activity. These values and those of the concentration of EP in Table 1 allowed to calculate turnover numbers that were 1964 min^{-1} for the control and 5215 min^{-1} for the Ca^{2+}-ATPase treated with Mg^{2+} plus vanadate. After this, it was clear that activation of the Ca^{2+}-ATPase by Mg^{2+} plus vanadate was due to changes in the kinetic properties of the enzyme rather than to activation of non-active enzyme units.

The results reported here show that pretreatment with Mg^{2+} plus vanadate increased the rate of phosphorylation of the Ca^{2+}-ATPase by ATP without changing the steady-state level of EP. For this level to remain the same, vanadate should also increase the rate of dephosphorylation. Furthermore, from what is known of the partial reactions of the Ca^{2+}-ATPase, the rate of ATP hydrolysis is limited by the reaction of conversion between conformers E_2 and E_1, whose k_{app} is near 3 s^{-1} [7]. If this were so, a higher Ca^{2+}-ATPase activity after preincubation with vanadate should be associated to a higher rate of the reaction E_2-E_1. This should result into accumulation of the E_1 conformer which in turn will lead to a faster phospho-

Table 1
Kinetic parameters of the Ca^{2+}-ATPase from human red cell membranes

Conditions	Steady-state level of EP (pmol/mg protein)	k_{app} (s^{-1})	Initial rate of phosphorylation (pmol/mg protein per s)	ATPase activity (pmol/mg protein per s)	Turnover number (min^{-1})
Control	1.9 ± 0.08 (12)	75 ± 11 (12)	141.4	62.2	1964
Pretreated	1.1 ± 0.11 (13)	244 ± 63 (13)	258.6	95.6	5215

ATPase activity was assayed as before [6] in media with the composition given in the legend to Fig. 1 for the media used during phosphorylation except that [γ-^{32}P]ATP was 100 μM and 0.1 mM ouabain was added. The values represent the difference between the amount of [^{32}P]orthophosphate liberated from [γ-^{32}P]ATP after 30 min incubation at 37° C in medium prepared with and without $CaCl_2$. The results shown are the mean value from three experiments run by triplicate. Steady state phosphorylation level (EP) and phosphorylation rate constant (k_{app}) were calculated by non-linear regression analyses of data presented in Fig. 1. The initial rate of phosphorylation was calculated as the product of k_{app} times the corresponding steady-state level of EP. Turnover number was the ratio between ATPase activity and the corresponding steady-state level of EP. Where indicated, mean values ± 1 S.E. are shown, with the degree of freedom included within parenthesis.

rylation [7]. Although this interpretation could be submitted to experimental test, no attempts were made during this study because to measure rates of dephosphorylation [8] and E_2 to E_1 conversion [7] in Ca^{2+}-ATPase from human red cell membranes at 37° C is quite difficult. Nevertheless, circumstantial support for the view mentioned above comes from our previous demonstration that pretreatment with Mg^{2+} plus vanadate renders Ca^{2+}-ATPase whose behaviour resembles that of the enzyme associated to calmodulin [3], and that partial proteolysis of the Ca^{2+}-ATPase (which mimics calmodulin in its effects) increases the rate of phosphorylation by favouring the E_1 conformation of the enzyme [7]. This allows to suggest therefore that, together with the increase in rate of phosphorylation and in turnover number, preincubation with Mg^{2+} plus vanadate could had stimulated also both dephosphorylation and the E_2 to E_1 conversion that take place during the reaction cycle of the Ca^{2+}-ATPase. Although results in this paper show that Mg^{2+} plus vanadate changed the catalytic properties of the Ca^{2+}-ATPase, they do not allow to draw any conclusion on the reactions leading to such change.

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