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TWO CLASSES OF SITE FOR ATP IN THE Ca^{2+}-ATPase FROM HUMAN RED CELL MEMBRANES

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Summary

(1) The response of the Ca^{2+}-ATPase activity from human red cell membranes to ATP concentrations can be represented by the sum of two Michaelis-like curves: one with a K_m of 2.5 μM and the other with a K_m of 145 μM.

(2) The maximum Ca^{2+}-ATPase activity elicited by occupation of the site with lower K_m represents about 10% of the activity attainable at non-limiting ATP concentrations.

(3) 30—50% of the Ca^{2+}-ATPase activity with lower K_m remains in the absence of Mg^{2+}. Mg^{2+} increases V and the maximum effect of Ca^{2+}, having no effect on the apparent affinities for ATP and Ca^{2+}.

(4) The large increase in Ca^{2+}-ATPase activity which results from the occupation of the site with higher K_m only takes place when Mg^{2+} is present.

(5) Results are compatible with the idea that the Ca^{2+}-ATPase from human red cell membranes has two classes of site for ATP binding, both of which are occupied when the enzyme catalyzes the hydrolysis of ATP at maximum rate.

(6) The properties of the high affinity site suggest that this is the catalytic site of the Ca^{2+}-ATPase. It is proposed that binding of ATP at the low affinity site regulates the turnover of the system.

Introduction

In the course of our studies on the phosphorylation and dephosphorylation reactions which take place during the hydrolysis of ATP catalyzed by Ca^{2+}-ATPase of human red cell membranes, we found that the concentration of ATP for half-maximal phosphorylation ranged from 1 to 6 μM [1]. This value is considerably lower than the Michaelis constant for the Ca^{2+}-ATPase reaction,

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Abbreviation: EGTA, ethyleneglycol bis (β-aminoethylether)-N,N'-tetraacetic acid.

reported to be within the range of 40—60 μM [2,3]. We also found that, in contrast with the strict requirement for Mg^{2+} of the overall Ca^{2+}-ATPase reaction [4], phosphorylation proceeds in the absence of Mg^{2+} and under these conditions a slow enzymatic hydrolysis of the phosphoenzyme takes place [1].

A possible cause for these discrepancies lies in the fact that, whereas phosphorylation is measured using micromolar concentrations of ATP, Ca^{2+}-ATPase activity is usually measured at concentrations of the nucleotide above 0.1 mM and in the presence of Mg^{2+}.

This paper reports experiments in which Ca^{2+}-ATPase activity and its dependence on Mg^{2+} was studied at ATP concentrations ranging from $5 \cdot 10^{-4}$ to 4 mM.

Materials and Methods

Materials. Fragmented membranes from human red cells were prepared as described previously [5]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [6] except that no unlabelled orthophospate was added to the incubation mixture.

^{32}P-labelled orthophosphate was provided by the Comisión Nacional de Energía Atómica (Argentina). ATP, enzymes and co-factors used for the synthesis of [γ-^{32}P]ATP were from Sigma Chemical Co., U.S.A. Other reagents were of analytical reagent grade.

Methods. Except were otherwise indicated, ATPase activity was measured in media containing 1 mM $MgCl_2$, 0.5 mM EGTA, 0.7 mM $CaCl_2$, 150 mM Tris · HCl, pH 7.8 at 37°C and various amounts of ATP and fragmented membranes. Ca^{2+}-ATPase activity was taken as the difference between the activity measured in the above media and the activity measured in media in which $CaCl_2$ was omitted.

When the concentration of ATP during the assays was higher than 0.1 mM, the nucleotide was added with an equimolar amount of $MgCl_2$. The concentration of protein from fragmented membranes was 1 mg/ml of medium and the inorganic phosphate liberated from ATP after incubation at 37°C for 30 min was estimated by a modification of the Fiske-Subba Row procedure [7]. All determinations were performed in triplicate. For ATP concentrations less than 0.1 mM [γ-^{32}P]ATP was used and the concentration of fragmented membranes was 0.1 mg membrane protein/ml medium. After 10 min incubation at 37°C, the tubes containing 0.3 ml of the reaction mixture were transferred to an ice-water bath and after 1 min each of them received 0.6 ml of isobutanol followed by 0.75 ml $(NH_4)MoO_4$, 0.5% (w/v) in 5% (v/v) perchloric acid. The mixture was vigorously stirred for 20 s and then spun down for 3 min at 1700 × *g*. The radioactivity in an aliquot of the organic phase was measured in a liquid scintillation counter and from this value the amount of inorganic phosphate liberated from ATP was calculated. All determinations were performed in quintuplicate. Under the conditions described no more than 10% of the ATP in the reaction mixture underwent enzymatic hydrolysis and the rate of appearance of inorganic phosphate remained constant up to 20 min incubation time.

The specific activity of the γ-phosphate of ATP was assumed to remain constant during the assay, in spite of the adenylate kinase activity of the red cell membranes [8,9] which tend to distribute the label between the β- and γ- phosphate of ATP. The assumption seems to be justified since the published K_m values [10] of the adelynate kinase for ATP (0.3—0.4 mM), ADP (0.18—1.6 mM) and AMP (0.07—2.7 mM) are far above the concentrations of these nucleotides expected in the assay media.

Solubilization of membranes was accomplished with Triton X—100 using a modification of the procedure of Wolf and Geitzen [11]. Fragmented membranes (3.5 mg protein/ml) were incubated 10 min at 0°C in 15 mM Tris · HCl (pH 7.4 at 0°C) with 0.4% (v/v) of Triton X-100. The suspension was then centrifuged at 17 000 × *g* for 30 min and the clear supernatant was used as the enzyme source. The protein concentration was measured according to Lowry et al. [12].

Results

Dependence of Ca^{2+}-ATPase activity on ATP concentration

Ca^{2+}-dependent ATPase was measured as a function of ATP concentration. Results (Fig. 1A) show that the response of Ca^{2+}-ATPase activity to ATP concentration is biphasic. The curve increases slowly and tends toward a plateau at ATP concentrations near 10 μM. Between 20 and 150 μM there is a large increase in activity and as ATP concentration exceeds 1 mM the curve tends again toward saturation. Reciprocal plots of ATPase activity against ATP concentration in the 0.5—16 μM ATP concentration range (Fig. 1B) and in the 0.125—4 mM ATP concentration range (Fig. 1C) yield straight lines. The cuve in Fig. 1 can, therefore, be represented by the sum of two Michaelis-like equations: one with high affinity for ATP and low maximum rate (Fig. 1B) and the other with low affinity and high maximum rate (Fig. 1C). The substrate curve of Ca^{2+}-ATPase activity therefore appears to be the sum of the effects that result from the binding of ATP at two non-equivalent sites.

The existence and the properties of the low and high affinity sites in the Ca^{2+}-ATPase were confirmed in six independent experiments whose results are summarized in Table I. It can be seen that there is a 55-fold difference in the apparent affinity for ATP between the low and high affinity sites. Although a rather large variation was observed, on the average there is a 10-fold difference between the rate of ATP hydrolysis due to occupation of the high affinity site, as compared to the activity at non-limiting ATP concentrations.

Sealing of the membranes or damage of the enzyme during isolation of the membranes could result in a population of the enzyme having low apparent affinity for ATP. To eliminate this possibility the effects of concentrations of ATP between 0.25 and 4 mM on Ca^{2+}-ATPase activity were measured on the membrane preparation used in these studies; on membrane isolated following the procedure of Schatzmann [13], which this author considers less damaging than ours; and in membranes solubilized with Triton X-100, a treatment that should abolish sealing. Results in Fig. 2 make it clear that for the three preparations the increase from 0.25 to 4 mM in the concentration of ATP results in a 2—3-fold increase of Ca^{2+}-ATPase activity. It seems, therefore, that the low affinity site for ATP is not an artifact caused by the preparation of membranes.

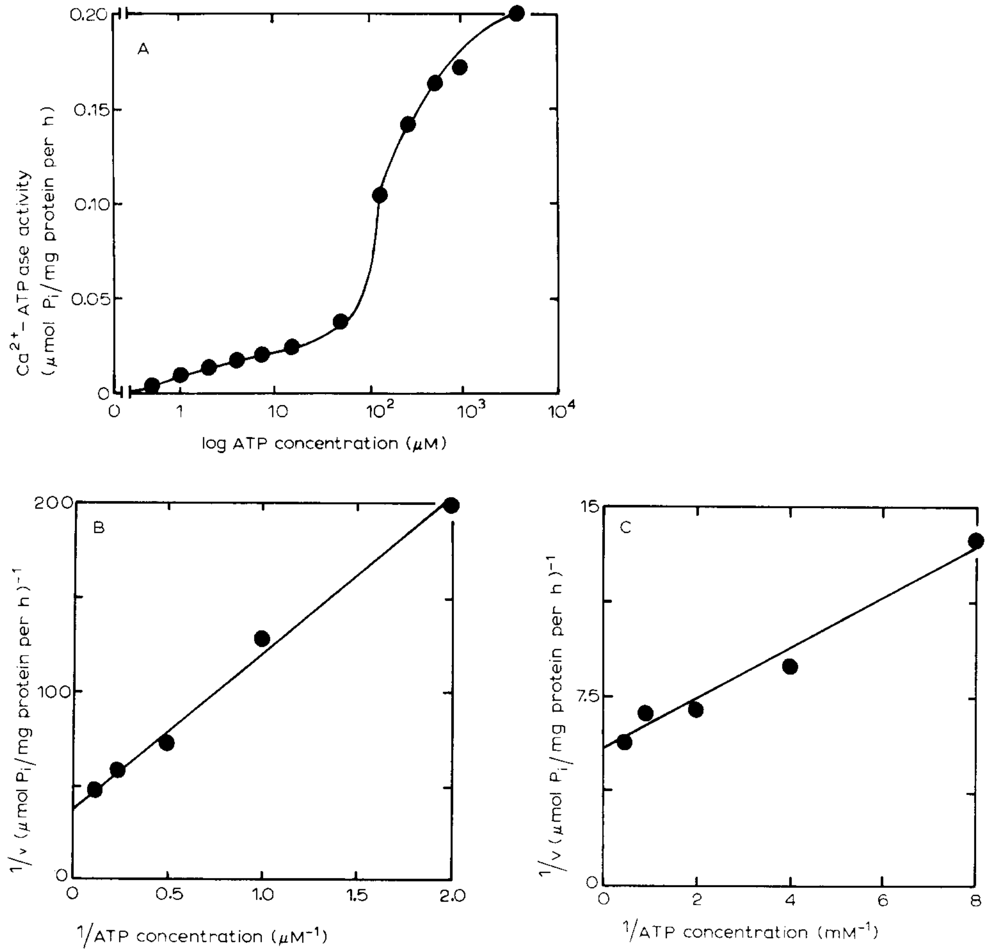


Fig. 1. (A) Ca^{2+}-ATPase activity as a function of ATP concentration. (B) Reciprocal plots of the values of Ca^{2+}-ATPase activity from A against ATP concentrations in the 0.5—16 μM concentration range. (C) Reciprocal plots of the values of Ca^{2+}-ATPase activity from A against ATP concentrations in the 0.125—4 mM ATP concentration range. The value of the maximal velocity calculated from the plot in B was subtracted from each of the values of Ca^{2+}-ATPase activity found in this ATP concentration range.

The effects of Mg^{2+}

It is generally accepted that both Ca^{2+}-ATPase activity [4] and active Ca^{2+} transport [14] in red cell membranes requires the presence of Mg^{2+}. The experimental evidence supporting this comes from experiments using ATP concentrations high enough to fully occupy the low affinity site described in the previous section. It seemed, therefore, interesting to study the effects of Mg^{2+} at low concentrations of ATP in order to assess the requirement for Mg^{2+} of the Ca^{2+}-ATPase under conditions in which only the high affinity site is occupied by the nucleotide. In the experiment of Fig. 3, Ca^{2+}-ATPase activity was measured at ATP concentrations up to 15 μM in media with and without Mg^{2+}. It

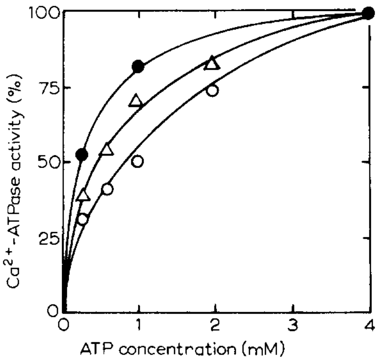
TABLE I

KINETIC PARAMETERS OF Ca^{2+}-ATPase ACTIVITY MEASURED AT TWO DIFFERENT CONCENTRATION RANGES OF ATP

Figures are the mean ± S.E. $n = 6$.

ATP concentration range (mM)	V (μmol/mg protein per h)	K_m (mM)
$0.5 \cdot 10^{-3}$—$16 \cdot 10^{-3}$	0.0343 ± 0.008	0.00246 ± 0.00068
0.125—4.0	0.375 ± 0.062	0.143 ± 0.0186

can be seen that there remains a substantial amount of Ca^{2+}-ATPase activity in the absence of Mg^{2+}. Reciprocal plots of the data in Fig. 3 demonstrated that Mg^{2+} only acts on the maximum velocity, having no effect on the K_m of the enzyme for ATP. The effect of Mg^{2+} was also tested on the relation between Ca^{2+} concentration and ATPase activity measured at low (10 μM) ATP concentrations. A reciprocal plot of such an experiment is shown in Fig. 4. Here again, Mg^{2+} only increases the maximum stimulatory effect of Ca^{2+}, having no effect on the concentration of Ca^{2+} for half-maximal activation of the ATPase. Fig. 5 shows the results of an experiment in which Ca^{2+}-ATPase activity was measured at non-limiting concentrations of Ca^{2+} and at 10 μM ATP as a function of Mg^{2+} concentration. It can be seen that the activity increases with Mg^{2+} along a curve that tends to saturate at approx. 1 mM Mg^{2+}. The reciprocal plot of the Mg^{2+}-dependent activity (see inset to Fig. 5), shows that the effect of Mg^{2+} can be fitted by a rectangular hyperbola that half-saturates at 0.33 mM Mg^{2+}. In other



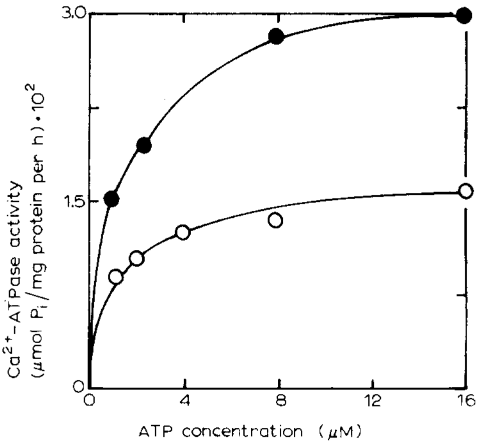
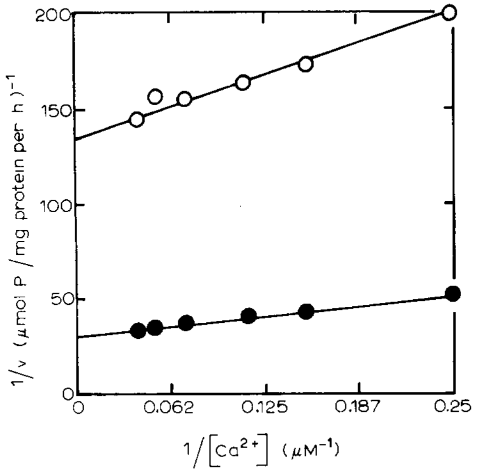


Fig. 2. Ca^{2+}-ATPase activity as a function of ATP concentration in fragmented membranes prepared according to the procedure of Garrahan et al. [5] (△), in fragmented membranes prepared by the procedure described by Schatzmann [13] (●) and in membranes solubilized with Triton X-100 (○). Results are expressed taking as 100% the activity at 4 mM ATP of each preparation of membranes.

Fig. 3. Ca^{2+}-ATPase activity as a function of ATP concentration in media with (●) and without (○) 1 mM $MgCl_2$.



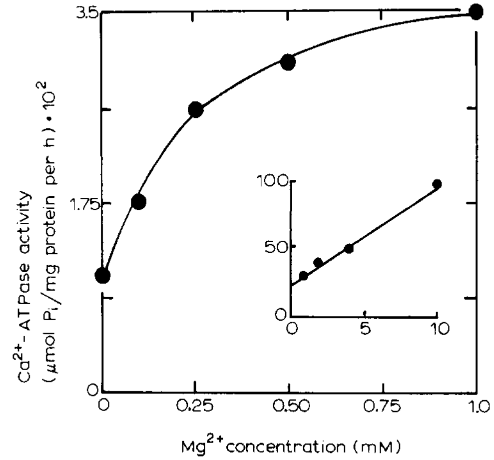


Fig. 4. A reciprocal plot of Ca^{2+}-ATPase activity as a function of Ca^{2+} concentration in media with (●) and without (○) 1 mM MgCl$_2$. The concentration of ATP was 10 μM. From the intercepts at the abscissa the concentration of Ca^{2+} for half-maximal activation can be calculated to be 2 μM in the absence and 2.6 μM in the presence of Mg^{2+}.

Fig. 5. Ca^{2+}-ATPase activity as a function of MgCl$_2$ concentration. The concentration of ATP was 10 μM. The inset shows a reciprocal plot of the activity in the presence of MgCl$_2$ minus the activity in the absence of MgCl$_2$ (mg protein per h per μmol P$_i$) as a function of MgCl$_2$ concentration mM^{-1}. From the intercept in the abscissa, the concentration of MgCl$_2$ for half-maximal activation can be calculated to be 0.33 mM.

experiments (not shown) Ca^{2+}-ATPase activity was measured at low (15 μM) and at high (2 mM) ATP concentrations in media with and without 2 mM MgCl$_2$. Results indicated that the large increase in ATPase activity observed between 15 μM and 2 mM ATP only took place if Mg^{2+} was also present. Hence, Ca^{2+}-ATPase activity is much more dependent on Mg^{2+} when it is tested in media with high rather than low ATP concentrations.

Discussion

Results presented in this paper show that, as judged by the effects of ATP on Ca^{2+}-ATPase activity, the red cell membrane possesses two classes of site at which ATP binds to elicit this activity: one has a high affinity for ATP (K_m = 2.5 μM) and the other has a low affinity for ATP (K_m = 145 μM). The simplest explanation for the existence of two classes of site is to assume that both are present in the Ca^{2+}-ATPase and to obtain a maximum rate of Ca^{2+}-dependent ATP hydrolysis both have to be occupied.

The properties of the high affinity site strongly suggest that this is the site at which ATP binds to transfer its terminal phosphate to the Ca^{2+}-ATPase. Three lines of evidence give support to this view, i.e., (i) the K_m of the high affinity site is almost identical to the concentration of ATP for half-maximal phosphorylation [1]; (ii) at low ATP concentrations half-maximal activity and half-maximal phosphorylation are reached at the same Ca^{2+} concentration; and (iii)

Ca^{2+}-dependent phosphorylation takes place in the absence of Mg^{2+} and, under these conditions, the phosphorylated intermediate undergoes enzymatic hydrolysis at a low rate [1]. In agreement with this, Ca^{2+}-ATPase activity persists at a low rate in the absence of Mg^{2+}. Therefore, it may be concluded that Ca^{2+}-ATPase observed at low ATP concentrations results from the occupation by ATP of the catalytic site of the Ca^{2+} pump.

In a recent publication [9], Schatzmann reported experiments in which Ca^{2+}-ATPase from human red cells was measured in a 10—200 μM ATP concentration range in the presence of Mg^{2+}. From these experiments and assuming that free ATP is the substrate, the author calculated a K_m value close to 1 μM. This value agrees with the K_m of the high affinity site reported in this paper from direct measurements of Ca^{2+}-ATPase activity in the absence of Mg^{2+}.

The maximum rate of ATP hydrolysis attainable by occupation of the high affinity site is far below that necessary to account for the observed maximum rate of active Ca^{2+} transport [14,15]. This makes it necessary to consider the low affinity site for ATP as a part of the Ca^{2+} pump, since only when the Ca^{2+}-ATPase activity which results from the occupation of this site is considered are the values for the stoicheiometry of Ca^{2+} transport [13,16] in red cells thermodynamically sound.

In the $(Na^+ + K^+)$-ATPase there is good evidence demonstrating the existence of high affinity sites for ATP identifiable with the catalytic site, together with a low affinity site at which ATP binds acting as an activator (see ref. 17). Biphasic curves for ATP activation have also been reported for the Ca^{2+}-ATPase from sarcoplasmic reticulum [18]. In this system detailed analysis of the concentration dependence of the rate of the Ca^{2+}-ATPase also demonstrated the need of ATP binding at a second site from which the nucleotide influences the rate of the overall reaction [19]. In view of this it is tempting to propose an activating role for ATP in the low affinity site of the Ca^{2+}-ATPase from red cells. Experimental evidence presented here, however, does not allow us to discard the possibility that this site shares the catalytic properties of the high affinity site.

When only the high affinity site is occupied by ATP, Mg^{2+} increases both the maximum velocity and the maximum effect of Ca^{2+}, having no effect on the apparent affinity of the ATPase for either ATP or Ca^{2+}. Experimental evidence therefore strongly suggests that the effects of Mg^{2+} are excerted at a site that is independent of those for ATP and for Ca^{2+}. This implies that, as we have already suggested [1], ATP rather than the $Mg^{2+} \cdot$ ATP complex is the actual substrate for the Ca^{2+}-ATPase at its high affinity site. Additional support for this view comes from the finding that the effects of Mg^{2+} are half-maximal at about 0.3 mM free Mg^{2+}, a value which exceeds 20 times the concentration of ATP needed for full occupation of the high affinity site. This result is difficult to conciliate with the idea that the effect of Mg^{2+} is the result of the formation of a $Mg^{2+} \cdot$ ATP complex.

In contrast with the partial dependence of Ca^{2+}-ATPase on Mg^{2+} at the high affinity site, the effect of ATP at the low affinity site is almost completely dependent on Mg^{2+}. It is this dependence that probably accounts for the strict requirement for Mg^{2+} of the overall Ca^{2+}-ATPase [4] and the coupled Ca^{2+} transport [14].

Acknowledgements

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