Biochimica et Biophysica Acta 858 (1986) 21–30
Elsevier

BBA 73103

The activation of phosphatase activity of the Ca^{2+}-ATPase from human red cell membranes by calmodulin, ATP and partial proteolysis

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(Received November 26th, 1985)

Key words: Ca^{2+} transport; Ca^{2+}-ATPase; Phosphatase activity; Calmodulin; (Erythrocyte membrane)

(1) Depending on the assay conditions, the ability of the Ca^{2+}-ATPase from intact human red cell membranes to catalyze the hydrolysis of *p*-nitrophenylphosphate is elicited by either calmodulin or ATP. The response of the phosphatase activity to *p*-nitrophenylphosphate, ATP, Mg^{2+} and K^+ is the same for the activities elicited by ATP or by calmodulin, suggesting that a single process is responsible for both activities. (2) In media with calmodulin, high-affinity activation is followed by high-affinity inhibition of the phosphatase by Ca^{2+} so that the activity becomes negligible above 30 μM Ca^{2+}. Under these conditions, addition of ATP leads to a large decrease in the apparent affinity for inhibition by Ca^{2+}. (3) In membranes submitted to partial proteolysis with trypsin, neither calmodulin nor Ca^{2+} are needed and phosphatase activity is maximal in media without Ca^{2+}. This is the first report of an activity sustained by the Ca^{2+}-ATPase of red cell membranes in the absence of Ca^{2+}. Under these conditions, however, ATP still protects against high-affinity inhibition by Ca^{2+}. These results strongly suggest that during activation by calmodulin, Ca^{2+} is needed only to form the calmodulin-Ca^{2+} complex which is the effective cofactor. (4) Protection by ATP of the inhibitory effects of Ca^{2+} and the induction of phosphatase activity by ATP + Ca^{2+} suggests that activation of the phosphatase by Ca^{2+} in media with ATP requires the combination of the cation at sites in the ATPase. (5) Results can be rationalized assuming that E_2, the conformer of the Ca^{2+}-ATPase, is endowed with phosphatase activity. Under this assumption, either the calmodulin-Ca^{2+} complex or partial proteolysis would elicit phosphatase activity by displacing the equilibrium between E_1 and E_2 towards E_2. On the other hand, ATP + Ca^{2+} would elicit the activity by establishing through a phosphorylation-dephosphorylation cycle a steady-state in which E_2 predominates over other conformers of the ATPase.

Introduction

Previous work from this laboratory showed that, like the other cation-transport ATPases, the Ca^{2+}-ATPase of human red cell membranes possesses a phosphatase activity towards *p*-nitrophenylphosphate [1]. Since we were only able to detect this activity in media with ATP, we proposed that the phosphatase of the Ca^{2+}-ATPase was strictly dependent on the nucleotide. In further studies [2], we provided experimental results in support of the idea that the active site for *p*-nitrophenylphosphate is the low-affinity, regulatory site for ATP of the ATPase and that for phosphatase activity ATP has to bind to the high-affinity, catalytic site of the enzyme.

Lucas et al. [3] reported that the phosphatase activity of red cell membranes was stimulated by Ca^{2+} and a crude preparation of calmodulin in media without ATP. Verma and Penniston [4] assayed the phosphatase activity of a purified and solubilized Ca^{2+}-ATPase preparation and concluded that in addition to the ATP-dependent activity, the enzyme was able to catalyze *p*-nitro-

phenylphosphate hydrolysis in the absence of the nucleotide in a way that was strictly dependent on calmodulin. These authors did not analyze in detail whether the ATP-dependent and the calmodulin-dependent activities are or not the expression of the same phenomenon.

This paper reports the results of experiments performed with the aim of reexamining the role of Ca^{2+}, ATP and calmodulin in the phosphatase activity of the Ca^{2+}-ATPase of red cell membranes. Results are consistent with the idea that a single state of the Ca^{2+}-ATPase is responsible for the phosphatase activity but that the mechanism of activation by Ca^{2+} depends on whether ATP or calmodulin are the promoters of this activity, the main difference being that calmodulin-dependent phosphatase requires the binding of Ca^{2+} to calmodulin whereas ATP-dependent phosphatase requires the binding of Ca^{2+} to the ATPase.

Materials and Methods

Fresh blood from hematologically normal adults collected on acid/citrate/dextrose solutions was always used. Calmodulin-depleted membranes were obtained by the procedure of Gietzen et al. [5].

Inside-out vesicles were prepared according to the procedure of Sarkadi et al. [6]. For treatment with trypsin, the membranes were washed and suspended in a medium containing: 30 mM Tris-HCl (pH 7.40 at 37°C), 60 μM $CaCl_2$ and 3 μg/ml trypsin (209 units/ml). The mixture was incubated at 37°C and after 3 min, trypsin action was terminated by the addition of a 50-fold (w/w) excess of soybean trypsin inhibitor plus 100 μM EGTA. Control experiments (not shown) indicated that after this treatment, the remaining Ca^{2+}-ATPase activity was about 70% of the initial and was insensitive to activation by calmodulin.

Phosphatase activity was measured estimating the release of *p*-nitrophenol from *p*-nitrophenylphosphate [1]. Except when otherwise indicated in Results, the incubation media contained: 120 mM KCl, 5.5 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.40 at 37°C), 10 mM *p*-nitrophenylphosphate, 1.0 mM EGTA, 0 or 0.5 mM ATP, 0 or 120 nM calmodulin, 1 mM ouabain, 50–100 μg/ml membrane protein and various concentrations of $CaCl_2$. ATPase activity was measured from the release of $[^{32}P]P_i$ from [γ-^{32}P]ATP [7] in media of similar composition as that used to estimate phosphatase activity, except that they contained various concentrations of ATP. Ca^{2+}-dependent activities were calculated from the difference between the activities in media with and without $CaCl_2$. Ca^{2+} uptake into inside-out vesicles was measured estimating the initial rate of $^{45}Ca^{2+}$ uptake in media of composition similar to those used to estimate the enzymic activities, the concentration of free Ca^{2+} was 1.25 μM and the concentration of vesicles was that equivalent to about 100 μg/ml of membrane protein. All incubations were carried out at 37°C.

The concentration of free Ca^{2+} in the incubation media was measured with an IS-561 Ca^{2+}-selective electrode [8]. Protein was estimated by the method of Lowry et al. [9].

Calmodulin was purified from bovine brain as described by Kakiuchi et al. [10]. [γ-^{32}P]ATP was prepared according to the procedure of Glynn and Chappell [11], except that no unlabelled orthophosphate was added to the incubation media. [^{32}P]Orthophosphate and $^{45}CaCl_2$ were provided by the Comisión Nacional de Energía Atómica (Argentina). ATP, *p*-nitrophenylphosphate, enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were obtained from Sigma (U.S.A.). Trypsin was from Worthington (U.S.A.). Salts and reagents were of analytical reagent grade.

Measurement of enzymic activities were performed in triplicate. The individual values did not differ from the mean more than 10%. Theoretical equations were adjusted to the experimental results by least-squares nonlinear regression using the procedure of Gauss-Newton with optional damping. The concentration variable was assumed to have negligible error and the velocity variable was assumed to be homoscedastic. The program was run on a microcomputer with 14-digit precision (Rossi, R.C. and Garrahan, P.J., unpublished data).

Results

Effects of ATP and calmodulin on the response of the phosphatase activity to Ca^{2+}

In the experiment shown in Fig. 1, the phosphatase activity of calmodulin-depleted red cell

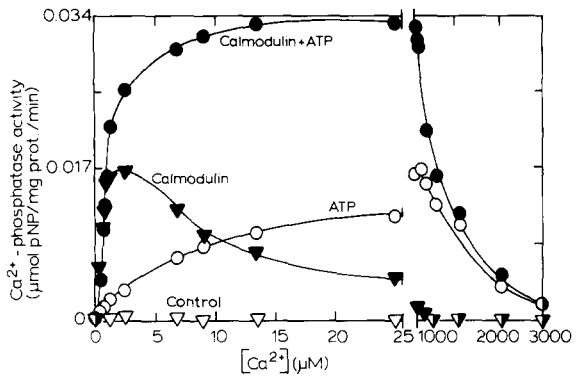

Fig. 1. Ca^{2+}-dependent *p*-nitrophenylphosphatase activity as a function of Ca^{2+} concentration in control media (▽); media with 120 nM calmodulin (▼); 0.5 mM ATP (○); or 0.5 mM ATP and 120 nM calmodulin (●). The concentration of free Ca^{2+} was measured in each of the media as indicated in Materials and Methods. The continuous lines are solutions of Eqn. 1 for the values shown in Table I. pNP, *p*-nitrophenol.

membranes was measured as a function of the concentration of Ca^{2+} in control media and in media with either calmodulin, ATP or ATP + calmodulin. In the absence of ATP and calmodulin, Ca^{2+} has no effect on the activity. In media with calmodulin and/or ATP, as Ca^{2+} concentration rises, the activity increases, passes through a maximum, and then tends to the level observed in the absence of Ca^{2+}. This response is adequately described by the equation:

$$v = \frac{V}{1 + \frac{K_{Ca}}{[Ca^{2+}]} + \frac{[Ca^{2+}]}{K_i}} \quad (1)$$

where v is the Ca^{2+}-dependent activity, K_{Ca} and K_i are apparent dissociation constants for Ca^{2+} as activator and inhibitor, respectively, and V is the value that v would reach at non-limiting concentrations of Ca^{2+} if the inhibitory component were absent. According to Eqn. 1, Ca^{2+}-dependent activity would tend to zero as the concentration of Ca^{2+} tends to infinity and maximum activity would be reached when:

$$[Ca^{2+}] = (K_{Ca} \times K_i)^{1/2} \quad (2)$$

Eqn. 1 was adjusted by non-linear regression to the experimental data of Fig. 1. The values of the parameters that gave best fit were used to draw the continuous curves in Fig. 1. These values, together with the calculated optimal rates are shown in Table I. In media with calmodulin alone, activation and inhibition by Ca^{2+} take place with high apparent affinity and the values of K_{Ca} and K_i are approximately equal. As a consequence of this, the activity reaches a narrow maximum, corresponding to 42% of the V, at 2.5 μM Ca^{2+} and becomes negligible at Ca^{2+} concentrations above 30 μM. As mentioned in the legend to Table I, to adjust Eqn. 1 to the data in media with calmodu-

TABLE I

KINETIC PARAMETERS OF THE EFFECTS OF Ca^{2+} ON PHOSPHATASE ACTIVITY

The best-fitting values and their standard errors were obtained by adjusting Eqn. 1 by nonlinear regression to the data in Fig. 1. In the experiment with calmodulin alone, the regression was performed keeping V fixed at a value similar to that obtained in the experiment with calmodulin + ATP. Almost identical values for V and K_i are obtained in this condition if K_{Ca} instead of V is fixed at the value for the experiment with calmodulin + ATP. The standard errors in the experiment with calmodulin alone are those that would have resulted if the values of the parameters had been obtained fitting the three parameters independently. Maximum activity was calculated inserting into Eqn. 1 the optimal concentration of Ca^{2+} calculated solving Eqn. 2 for the tabulated values of K_{Ca} and K_i. The percent of the activity at optimum Ca^{2+} concentration was calculated from the ratio between the activity at this concentration and the estimated value of V.

Additions		V	K_{Ca}	K_i	% Activity
calmodulin (nM)	ATP (μM)	(μmol/mg protein per min)	(μM)	(μM)	at optimum Ca^{2+} (v/V)
120	0	0.0315 ± 0.0149	1.60 ± 1.15	3.45 ± 2.37	42
120	500	0.0319 ± 0.0014	1.60 ± 0.23	319.4 ± 57.7	75
0	500	0.0182 ± 0.0013	17.62 ± 3.15	635.4 ± 118.3	88

lin alone, it was necessary to keep either V or K_{Ca} fixed at the values found in the experiment with calmodulin + ATP. If this is not done, since the values of K_{Ca} and K_i are close, good adjustments to the experimental data will be obtained for a wide range of individual values of these parameters, as long as their product satisfies Eqn. 2. As a consequence of this, the best-fitting values of the parameters will have such large standard errors as to deprive them of any practical meaning. The assumption that the values of K_{Ca} or of V of the experiment with calmodulin + ATP are good estimates of those in media with calmodulin alone seems reasonable in view that the initial part of the activation curves in both media are superimposable (Fig. 1).

When ATP and calmodulin are present together, high-affinity activation by Ca^{2+} persists but the nucleotide induces a substantial reduction in the apparent affinity for inhibition by Ca^{2+}. As a consequence of this, the activity vs. Ca^{2+} concentration curve reaches a broad maximum between 20 and 60 μM Ca^{2+}, and inhibition becomes noticeable only at Ca^{2+} concentrations above 100 μM. The best-fitting value of V for the experiment performed in media with calmodulin + ATP seems to be not significantly different from that of the experiment performed in media with calmodulin alone (Table I). However, since in media with ATP + calmodulin a much larger fraction (75%) of V can be expressed (Table I), the observable activity in the presence of ATP is higher than in its absence (Table I).

In media with ATP alone, K_{Ca} is about 10-times larger than in the presence of calmodulin. Under these conditions, the activity reaches a plateau above 150 μM Ca^{2+}, and inhibition becomes apparent only at Ca^{2+} concentrations higher than 250 μM. In this case, the best-fitting value of V is about half that in media with ATP + calmodulin or with calmodulin alone (Table I). In spite of this, since with ATP alone about 88% of V will be expressed, the optimal activity in this media is similar to that in media with calmodulin alone.

Fig. 1 also shows that at all the concentrations of Ca^{2+} tested, the rate measured in media with ATP + calmodulin is higher than the sum of the activities measured in media with either calmodulin or ATP.

Phosphatase activity after partial proteolysis

In the presence of calmodulin, the value of K_{Ca} for activation of the phosphatase is similar to the value of the equilibrium constant for the dissociation of Ca^{2+} from calmodulin [12]. In view of this, it is pertinent to ask if the requirement for Ca^{2+} of this activity expresses the requirement of Ca^{2+} for the formation of the active calmodulin-Ca^{2+} complex, rather than the combination of Ca^{2+} with the Ca^{2+}-ATPase.

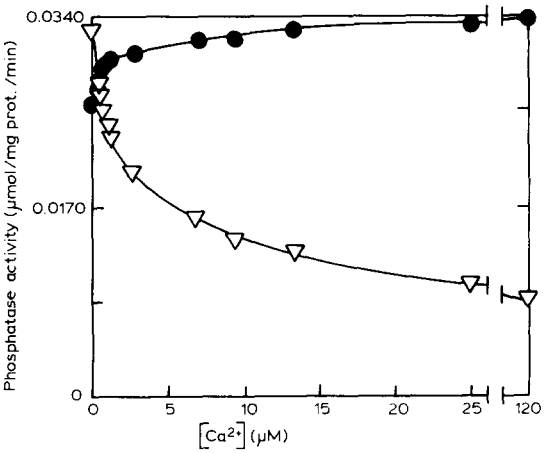

Fig. 2. Total *p*-nitrophenylphosphatase activity as a function of Ca^{2+} concentration in membranes submitted to controlled proteolysis with trypsin. Incubations were performed in media with (●) and without (▽) 0.5 mM ATP. Almost identical results were obtained if the incubation media contained 120 nM calmodulin (results not shown). The curve that fits the experiment with ATP is the solution of the equation:

$$v = V_0 + (V - V_0)/(1 + K_{0.5}/[Ca^{2+}]) \quad (3)$$

Eqn. 3 assumes that Ca^{2+} is a non-essential activator acting at identical and non-interaction sites. V_0 is the activity at 0 μM Ca^{2+}, V is the maximum activity and $K_{0.5}$ is the apparent dissociation constant for Ca^{2+}. The best-fitting values were: $V_0 = 0.0235$ μmol *p*-nitrophenol/mg protein per min; $V = 0.0314$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 0.570$ μM. The curve that fits the experiment without ATP is the solution of the equation:

$$v = V_0/(1 + [Ca^{2+}]/K_i) + V_r \quad (4)$$

Eqn. 4 assumes that Ca^{2+} is a partial inhibitor acting at identical and non-interacting sites. V_r is the activity insensitive to inhibition by Ca^{2+} and K_i is the concentration of Ca^{2+} for half-maximal inhibition. The best-fitting of the parameters were: $V_0 = 0.0201$ μmol *p*-nitrophenol/mg protein per min; $V_r = 0.0100$ μmol *p*-nitrophenol/mg protein per min and $K_i = 2.92$ μM.

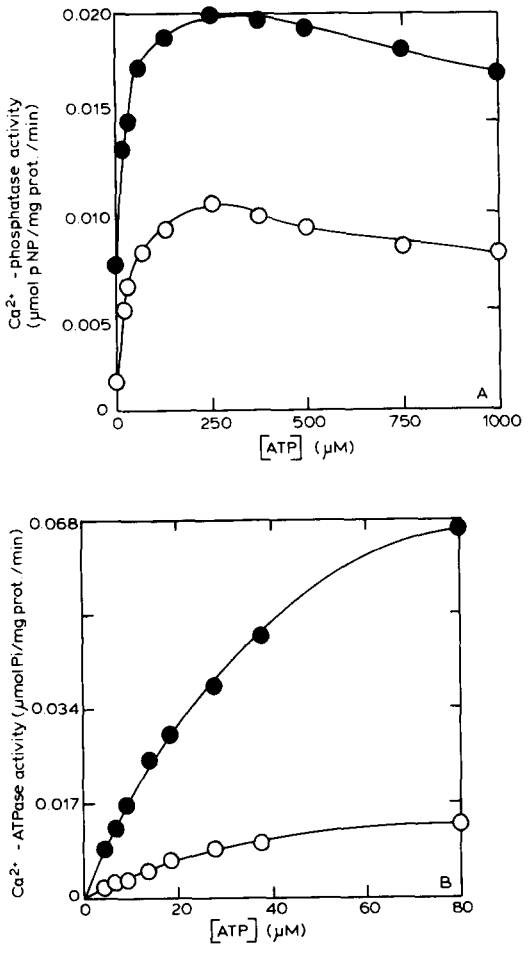

Fig. 3. (A) Ca^{2+}-dependent *p*-nitrophenylphosphate as a function of ATP in the presence (●) and in the absence (○) of 120 nM calmodulin. The continuous lines are the solutions of the equation:

$$v = \frac{V \cdot K_i \cdot [ATP] + V_0 \cdot K_m \cdot K_i}{[ATP]^2 + K_i[ATP] + K_m \cdot K_i} \quad (5)$$

Eqn. 5 has the same meaning as Eqn. (1), except that it assumes that there is activity (V_0) when the concentration of the ligand is zero. The best-fitting values of the parameters were: $V = 0.0235$ μmol *p*-nitrophenol/mg protein per min; $V_0 = 0.0090$ μmol *p*-nitrophenol/mg protein per min; $K_m = 45.47$ μM and $K_i = 3387$ μM for the experiment in the presence of calmodulin and $V = 0.0133$ μmol *p*-nitrophenol/mg protein per min; $V_0 = 0.0017$ μmol *p*-nitrophenol/mg protein per min; $K_m = 42.71$ μM and $K_i = 1643$ μM for the experiment in the absence of calmodulin. Incubations were performed in media with 10 mM *p*-nitrophenylphosphate and 6.8 μM Ca^{2+}. (B) Ca^{2+}-ATPase activity as a function of ATP in media containing 10 mM *p*-nitrophenylphosphate and 6.8 μM Ca^{2+} with (●) and without (○) 120 nM calmodulin. The continuous curve that fits the experiment with calmodulin is the solution of the equation:

$$v = V_1/(1 + K_{m1}/[ATP]) + [ATP] \cdot V_2/K_{m2} \quad (6)$$

for $V_1 = 0.00823$ μmol P_i/mg protein per min; $K_{m1} = 50.00$ μM and $V_2/K_{m2} = 0.00000523$ l/min per mg protein. The curve that fits the experiment without calmodulin is the solution of the equation:

$$v = V/(1 + K_m/[ATP]) \quad (7)$$

for $V = 0.00182$ μmol/mg protein per min and $K_m = 51.21$ μM. Since the substrate curve of the Ca^{2+}-ATPase can be described as the sum of two Michaelis-Menten-like components [7], the second term in the right-hand-side of Eqn. 6 represents the initial, linear part of the component of lower apparent affinity. This component was not taken into account in Eqn. 7 because, as described by Rossi et al. [19], in the absence of calmodulin the contribution of this component is negligible at the low concentrations of ATP tested. pNP, *p*-nitrophenol.

It is known that controlled proteolysis of membranes or of solubilized enzyme with trypsin [13,14], in the presence of Ca^{2+} [15], drives the Ca^{2+}-ATPase into a state that is functionally equivalent to that attainable with calmodulin plus Ca^{2+}. Hence, if activation by Ca^{2+} expressed the requirements for Ca^{2+} of calmodulin, after membranes are submitted to controlled proteolysis with trypsin, the cation would be no longer necessary for phosphatase activity. This prediction was submitted to experimental test studying the response to Ca^{2+} of the total phosphatase activity of membranes submitted to controlled proteolysis. Results in Fig. 2 show that under these conditions and in the absence of ATP, the phosphatase activity is maximal in Ca^{2+}-free media and then decrease as the concentration of Ca^{2+} rises. The concentration of Ca^{2+} for half-maximal inhibition is 2.92 μM. When ATP is present, there is a small drop in the activity at zero Ca^{2+}, the activity recovers as Ca^{2+} is increased reaching a value that remains constant up to 120 μM Ca^{2+}. This result is clear in demonstrating that ATP also protects the phosphatase activity from trypsin-treated membranes against the inhibition by Ca^{2+}.

It could be argued that after partial proteolysis the phosphatase activity of Ca^{2+}-ATPase is blocked and what Fig. 2 shows is the increase in the activity of a phosphatase unrelated to the Ca^{2+}-ATPase. This, however, seems to be unlikely,

since as shown in Fig. 2, after proteolysis the phosphatase activity is still inhibited by Ca^{2+} with the same high apparent affinity as in intact membranes incubated with calmodulin, and that ATP protects against this inhibition in the same way as it does in intact membranes. In view of this and of the known effects of partial proteolysis on the Ca^{2+}-ATPase, it seem reasonable to interpret the experiment in Fig. 2 as indicating that when Ca^{2+}-ATPase is in the 'phosphatase state', the only effect of Ca^{2+} is to inhibit.

The effect of ATP on phosphatase activity

In the experiment shown in Fig. 3A, Ca^{2+}-phosphatase activity was measured as a function of the concentration of ATP in media with 6.8 μM Ca^{2+}, in the presence and absence of calmodulin. It can be seen that in the two conditions tested, ATP increases Ca^{2+}-phosphatase activity with the same apparent affinity. Inhibition by high concentrations of ATP is seen both in the presence and in the absence of calmodulin and it is probably caused by competition between *p*-nitrophenylphosphate and ATP [2]. Results in Fig. 3A also show that for all the ATP concentrations tested, the sum of the activity due to calmodulin at 0 mM ATP and the activities measured in media with ATP and without calmodulin is always smaller than the corresponding activity in media with ATP + calmodulin.

Ca^{2+}-ATPase activity was measured as a function of the concentration of ATP in media of identical composition as those used in the experiment in Fig. 3A. Results in Fig. 3B show that the K_m of the initial part of the substrate curve is independent of the presence or absence of calmodulin and its value is very similar to that of the $K_{0.5}$ for activation of the Ca^{2+} phosphatase by ATP (Fig. 3A). This result extends to the phosphatase activity in media with calmodulin our previous finding [2] that enhancement of Ca^{2+} phosphatase by ATP takes place at a site with properties similar to those of the high-affinity site for ATP of the Ca^{2+}-ATPase.

The effects of calmodulin and ATP on the substrate curve

Ca^{2+}-phosphatase activity was measured as a function of *p*-nitrophenylphosphate concentration in media with and without calmodulin and with and without ATP. As we have described in detail elsewhere [2], the activity vs. *p*-nitrophenylphosphate concentration curve of the Ca^{2+} phosphatase can be adjusted by the equation:

$$v = \frac{V}{\left(1 + \frac{K}{[\text{pNPP}]}\right)^2} \qquad (8)$$

where V is the maximum velocity, K is an apparent dissociation constant, and pNPP is *p*-nitrophenylphosphate.

Results in Fig. 4 show that calmodulin increases the maximum rate but has no effect on the apparent affinity of the phosphatase for *p*-nitrophenylphosphate. The higher activity in the media with calmodulin plus ATP is predictable from the results in Fig. 1. On the other hand, the fact that K is larger in media with ATP is in keeping with the know competitive interactions between ATP and *p*-nitrophenylphosphate [2].

Activation by Mg^{2+}

Fig. 5 shows the result of an experiment in which Ca^{2+}-phosphatase activity was measured as a function of the concentration of Mg^{2+} in media with either calmodulin, ATP or ATP + calmodulin. Under all conditions tested, the response to Mg^{2+} is biphasic, activation being followed by inhibition. For this reason, an equation similar to Eqn. 1 was fitted to each of the experimental curves. Results makes clear that, although the maximum effect of Mg^{2+} changes in the different conditions tested, the apparent affinity for activation by Mg^{2+} remains unaffected by ATP or calmodulin. On the other hand, inhibition by Mg^{2+} seems to take place with higher apparent affinity if calmodulin is present in the incubation media.

Activation by potassium

In the experiment shown in Fig. 6, phosphatase activity was measured as a function of K^+ concentration in media containing calmodulin, with and without 0.5 mM ATP. It can be seen that both in the presence and in the absence of ATP, K^+ induces a strong activation of the phosphatase. Neither the apparent affinity for activation nor the maximum activation, judged by the ratio between the activity at non-limiting concentration

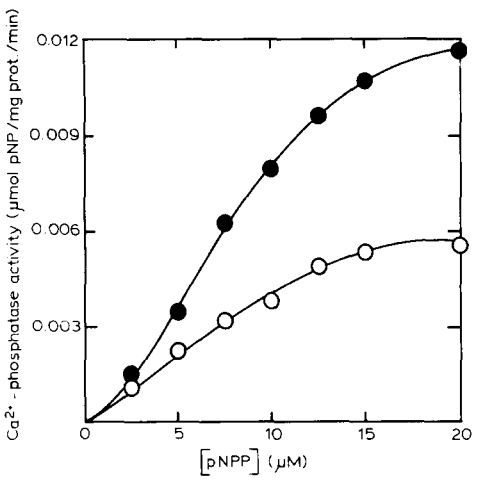

Fig. 4. Ca^{2+}-dependent *p*-nitrophenylphosphatase as a function of *p*-nitrophenylphosphate concentration in the presence of 120 nM calmodulin and 6.8 μM Ca^{2+} with (●) and without (○) 0.5 mM ATP. The continuous curves are solutions of Eqn. 3 with $V = 0.0209$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 6.35$ mM for the curve in the presence of ATP and $V = 0.0089$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 4.78$ mM for the curve in the absence of ATP. pNP, *p*-nitrophenol; pNPP, *p*-nitrophenylphosphate.

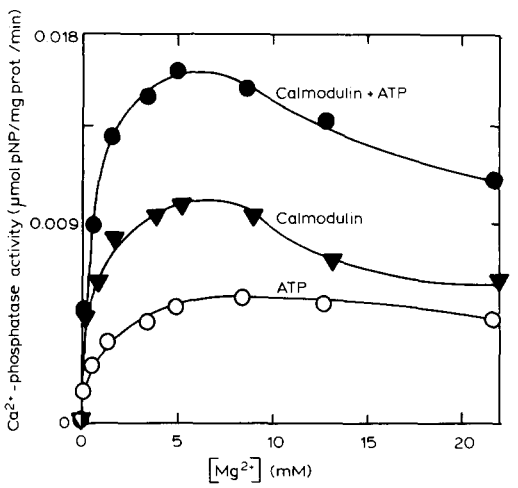

Fig. 5. Ca^{2+}-dependent *p*-nitrophenylphosphatase as a function of Mg^{2+} concentration in media with either 120 nM calmodulin and 0.5 mM ATP (●); 120 nM calmodulin (▼); or 0.5 mM ATP (○). The continuous curves are solutions of Eqn. 1 for: $V = 0.0190$ μmol *p*-nitrophenol/mg protein per min; $K_{0.5} = 0.78$ mM and $K_i = 28.73$ mM in the curve with calmodulin + ATP; $V = 0.0125$ μmol *p*-nitrophenol/mg protein per min; $K_{0.5} = 0.80$ mM and $K_i = 21.33$ mM in the curve with calmodulin alone and, $V = 0.0062$ μmol *p*-nitrophenol/mg protein per min; $K_{0.5} = 0.94$ mM and $K_i = 81$ mM in the curve with ATP alone. The experiments were performed in media with 2.5 μM Ca^{2+}.

and the activity at 0 mM K^+, are significantly different in the two conditions tested.

The activation of Ca^{2+} phosphatase by K^+ in media with ATP but without calmodulin was also tested. Results (not shown), indicated that under these conditions the apparent affinity for activation is similar to that observed in media with calmodulin but that the maximum effect of K^+ is smaller. A similar effect of calmodulin has been reported by Scharff [16] for the activation by K^+ of Ca^{2+}-ATPase activity.

Effect of phosphatase activity on active Ca^{2+} transport

We have reported experiments showing that the ATP-promoted hydrolysis of *p*-nitrophenylphosphate does not drive active transport of Ca^{2+} in reconstituted ghosts [1] or in inside-out vesicles [17]. The experimental conditions used made it mandatory to measure the effect of *p*-nitrophenylphosphate in the presence of ATP-dependent Ca^{2+}

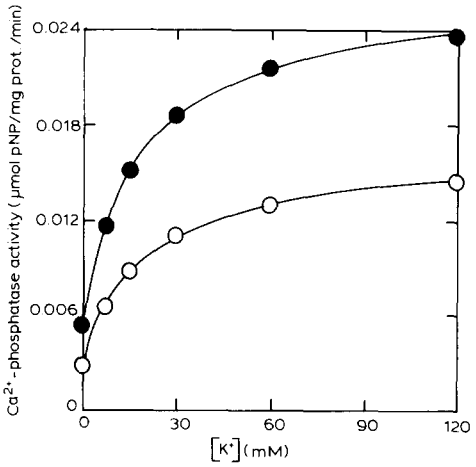

Fig. 6. Ca^{2+}-dependent *p*-nitrophenylphosphatase as a function of K^+ concentration in the presence (●) and the absence (○) of 0.5 mM ATP. The continuous curves are solutions of the equation (see legend to Fig. 2):

$$v = V_0 + (V - V_0)/(1 + K_{0.5}/[K^+]) \quad (9)$$

for $V = 0.0244$ μmol *p*-nitrophenol/mg protein per min, $V_0 = 0.0052$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 17.10$ mM for the curve in the presence of ATP and $V = 0.0157$ μmol *p*-nitrophenol/mg protein per min; $V_0 = 0.0032$ μmol *p*-nitrophenol/mg protein per min and $K_{0.5} = 18.94$ mM for the curve in the absence of ATP. The experiments were performed in media with 2.5 μM Ca^{2+}, pNP, *p*-nitrophenol.

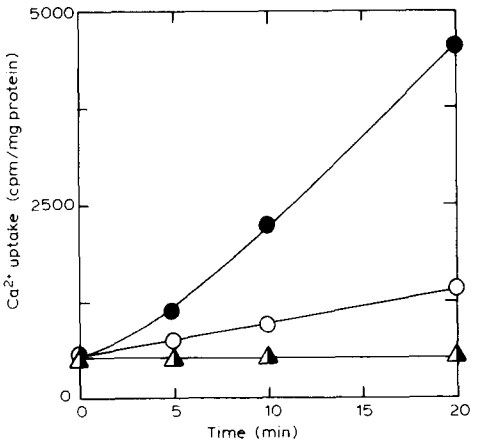

Fig. 7. Ca^{2+} uptake into inside-out vesicles as a function of time of incubation, in control media (△) and in media with 0.5 mM ATP (○), 120 nM calmodulin (▲) or 0.5 mM ATP and 120 nM calmodulin (●). Each measurement was performed by triplicate. The incubation media contained 1.25 μM Ca^{2+} and 10 mM *p*-nitrophenylphosphate.

transport. The experimental evidence therefore was indirect and depended on the comparison of the effects of *p*-nitrophenylphosphate on Ca^{2+}-ATPase activity and on ATP-dependent Ca^{2+} transport.

The availability of conditions for promoting phosphatase activity without ATP allows a direct examination of the role of *p*-nitrophenylphosphate as substrate for active Ca^{2+} transport. For this reason, the effects of ATP and of calmodulin on the rate of Ca^{2+} uptake by inside-out vesicles of red cell membranes in media containing 10 mM *p*-nitrophenylphosphate were measured. Results in Fig. 7 show that ATP promotes Ca^{2+} uptake and that while calmodulin increases 4.5-times the rate of Ca^{2+} uptake in the presence of ATP, it is without effect on the uptake in media with *p*-nitrophenylphosphate and without ATP. Extending previous findings, these results indicate that, under the conditions in which full expression of the Ca^{2+}-phosphatase activity is to be expected, no Ca^{2+} transport is detectable.

Discussion

Results in this paper show that when calmodulin is present, the phosphatase activity of the red cell membrane Ca^{2+}-ATPase can be detected in media without ATP. This agrees with the observations of Lucas et al. [3] and of Verma and Penniston [4] about the existence of a calmodulin-dependent phosphatase activity in preparations of red cell Ca^{2+}-ATPase incubated in medium without ATP. Therefore, the absolute requirement for ATP during phosphatase activity is not as we had previously proposed, a general feature of the Ca^{2+}-ATPase but a property of calmodulin-depleted preparations only.

Regardless of the condition used to elicit the activity, the apparent affinities of the phosphatase for activation by *p*-nitrophenylphosphate, ATP, Mg^{2+} or K^+ remain unchanged and under no condition *p*-nitrophenylphosphate hydrolysis is able to drive active transport of Ca^{2+}. The similarities between kinetic properties suggest that the same state of the Ca^{2+}-ATPase catalyzes phosphatase activity in media with calmodulin, ATP or ATP + calmodulin. If this view is taken for granted, the differences in the response of the phosphatase to Ca^{2+} induced by ATP and/or calmodulin would result from the different mechanisms by which these ligands drive the Ca^{2+}-ATPase into the state with phosphatase activity.

Activation by Ca^{2+}

Experiments in this paper show that Ca^{2+} is needed for phosphatase activity when the activity is elicited by ATP or calmodulin, but not when it is elicited by partial proteolysis. These results seem to indicate that Ca^{2+} participates in the process that drives the ATPase into the state with phosphatase activity but is not a direct cofactor in the catalysis of *p*-nitrophenylphosphate hydrolysis. Moreover, the demonstration that, in trypsin-treated membranes, full phosphatase activity is attainable in media without Ca^{2+} is, to the best of our knowledge, the first report of an activity of the Ca^{2+}-ATPase of plasma membranes that is apparent in the absence of Ca^{2+}.

Concerning the mechanism of the activation of the phosphatase by Ca^{2+}, results in this paper show that it differs markedly depending on whether ATP or calmodulin is the cofactor. In fact, when ATP is used, Ca^{2+} seems to be needed at its site(s) in the Ca^{2+}-ATPase. This view is supported both by our previous finding [1] that the apparent affinity for Ca^{2+} during activation of

the phosphatase is similar to that for activation of the ATPase and by the observation reported in this paper about the low apparent affinity of the phosphatase for Ca^{2+} in preparations depleted of calmodulin. Since the conditions for eliciting phosphatase activity with ATP are the same as those for phosphorylation of the Ca^{2+}-ATPase and since ATPase activity proceeds concomitantly with phosphatase activity [18], it seems reasonable to postulate that activation of the phosphatase by Ca^{2+} in the presence of ATP involves the formation of the phosphoenzyme of the Ca^{2+}-ATPase.

In media without ATP and with calmodulin, the role of Ca^{2+} appears to be substantially different, since activation of the phosphatase seems to require the binding of Ca^{2+} to calmodulin rather than to the ATPase. This assertion receives strong support from the experiments with membranes that had been submitted to partial proteolysis with trypsin, a procedure that mimics the effects of calmodulin, in which, as mentioned before, full activation of the phosphatase was attained in the absence of Ca^{2+} and the only observable effect of Ca^{2+} was to inhibit.

Inhibition by Ca^{2+}

The response of the phosphatase to Ca^{2+} at concentrations higher than those needed for activation, differs markedly depending on the presence or absence of calmodulin and/or ATP. In media with calmodulin alone, Ca^{2+} inhibits with high apparent affinity, preventing in this way the full expression of the catalytic capacity of the phosphatase. ATP increases the value of K_1 for Ca^{2+} so that the highest rates of phosphatase activity are attained in media with calmodulin + ATP. Protection by ATP explains why, at Ca^{2+} concentrations above 30 μM, phosphatase activity becomes completely dependent on the presence of the nucleotide. This fully accounts for the inability to find phosphatase activity in the absence of ATP in our previous studies, since in all of them we had measured phosphatase activity in media containing from 30 to 150 μM Ca^{2+} using membrane preparations that were partially depleted of calmodulin.

The mechanism of activation by calmodulin

Results in this paper could be interpreted as indicative that in the absence of ATP, the calmodulin-Ca^{2+} complex or the treatments that mimic its effect stabilize the Ca^{2+}-ATPase in a state endowed with phosphatase activity, whereas binding of Ca^{2+} to the enzyme drives it into a state devoid of such activity. Current experimental evidence indicates that, like the other cation-transport ATPases (for a review see Ref. 18) the Ca^{2+}-ATPase can exist in two conformational states, differing in functional properties, i.e., the E_1 conformer which possesses the high-affinity, catalytic site for ATP and the transport site for Ca^{2+}, and the E_2 conformer which possesses the low-affinity, regulatory site for ATP. In previous studies [2], we provided evidence that the active site for the phosphatase is the regulatory site for ATP of the Ca^{2+}-ATPase. As this site pertains to E_2, it seems reasonable to think that E_2 is the conformer endowed with phosphatase activity. If this is accepted, ligands that elicit phosphatase activity would increase the concentration of E_2 relative to E_1. Evidence favoring the idea that calmodulin stabilizes E_2 comes from experiments showing that the apparent affinity of the regulatory site for ATP of the ATPase tends to zero as membranes are deprived of calmodulin [19]. It seems, therefore, plausible to propose displacement of the equilibrium between E_1 and E_2 towards the latter as the mechanism by which calmodulin and tryptic digestion elicit phosphatase activity. Following the same line of thought, it seem reasonable to propose that stabilization of the ATPase in the E_1 conformer with the consequent decrease in the concentration of E_2 would explain why Ca^{2+} inhibits the phosphatase in media without ATP, and why the apparent affinity for this effect is similar to the apparent affinity for activation of the Ca^{2+}-ATPase by Ca^{2+}.

The mechanism of activation by ATP

The currently accepted view of the reaction cycle of the Ca^{2+}-ATPase implies that, when the ATPase is undergoing net turnover, the E_2 conformer can be reached through a series of steps involving the phosphorylation of E_1 by ATP to form E_1P, followed by the conversion of E_1P into E_2P and its subsequent dephosphorylation [18]. The existence of this pathway endows the Ca^{2+}-ATPase with a mechanism to 'escape' from the E_1

state, into which it would be driven by Ca^{2+} in the absence of ATP, and thus provides for a plausible explanation of the protection by ATP against inhibition by Ca^{2+}. Formation of E_2 after a phosphorylation-dephosphorylation cycle also seems to explain why, if Ca^{2+} is present, ATP becomes able to elicit phosphatase activity in calmodulin-depleted preparations.

The relative concentrations of E_1 and E_2

If we accept that phosphatase activity is a property of E_2, the lack of this activity in media without calmodulin or ATP implies that, under these conditions, a negligible fraction of the Ca^{2+}-ATPase is in the E_2 state and that binding of *p*-nitrophenylphosphate does not displace the Ca^{2+}-ATPase towards E_2. No experimental data about the relative concentrations of E_1 and E_2 in the resting ATPase is yet available. On the other hand, *p*-nitrophenylphosphate would alter the distribution between conformers, either if it reacted with the ATPase promoting a phosphorylation-dephosphorylation cycle of if it bound exclusively or preferentially to one of the conformers. It is not known whether *p*-nitrophenylphosphate replaces ATP in forming a phosphoenzyme able to undergo further transformations. Concerning direct effects of *p*-nitrophenylphosphate binding, if the affinities of E_1 and E_2 for this substrate were similar, their relative concentration would be independent of *p*-nitrophenylphosphate. Current experimental evidence suggests that *p*-nitrophenylphosphate binds equally well to E_1 and E_2. This is based on the observation that *p*-nitrophenylphosphate competes with similar apparent affinities both with the effects of ATP at the high-affinity site, which is thought to pertain to E_1, and, with the effects of ATP at the low-affinity site, which is thought to pertain to E_2 [18].

For the mechanisms involving phosphorylation by ATP to account both for protection against inhibition by Ca^{2+} and for phosphatase activity, the steady-state distribution among conformers during net turnover of the ATPase should be in favor of E_2. Although there is no direct information for or against this view, present experimental evidence seems to be consistent with it. In fact, it has been calculated by Schatzmann and Luterbacher [20] that during normal turnover only a small fraction of the ATPase is in the phosphorylated state. If this is taken together with the proposal by Muallem and Karlish [21] that, like in the other cation-transport ATPases, the $E_2 \rightarrow E_1$ transition is the rate-limiting step in the overall ATPase reaction [18], E_2 would be the predominant conformer during steady-state turnover of the ATPase.

Acknowledgments

This work was supported by grants from the CONICET and Universidad de Buenos Aires, Argentina. The authors are established investigators from the CONICET.

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