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SUBSTRATE SPECIFICITY OF THE ERYTHROCYTE Ca^{2+}-ATPase

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In the absence of Mg^{2+}, the observed activity of the erythrocyte plasma membrane Ca^{2+}-ATPase is due to the hydrolysis of CaATP at a low rate. In the presence of Mg^{2+}, the activity of the enzyme is much higher, but it is inhibited by high levels of free Mg^{2+}. This inhibition appears to be due to competition of Mg^{2+} and Ca^{2+} for a site on the enzyme, rather than for ATP.

Introduction

The nature of the energy-donating substrate of the red cell calcium pump has been a subject of controversy in the literature. Experimental findings have been interpreted to suggest the substrate of the pump was MgATP [1,2], free ATP [3–5] or CaATP [6,7]. The claim that free ATP is the substrate for the enzyme is based on the observation that the enzyme is capable of operating at a slow rate in the absence of Mg^{2+}. The belief that CaATP is the substrate is based on inhibition of the enzyme by Mg^{2+} in a manner which suggested that Mg^{2+} and Ca^{2+} were competing for ATP.

Materials and Methods

The isolation of the Ca^{2+}-ATPase, the assay of ATPase activity used for the purified enzyme, and the determination of protein were as previously described [7]. The molarity of EDTA and EGTA, $CaCl_2$, and $MgCl_2$ solutions were determined by titration.

Preparation of erythrocyte membrane vesicles: a preparation enriched in inside-out vesicles was prepared by standard methods [8,9]. However, the dextran gradient step utilized for separating inside-out from right-side-out vesicles was omitted, because it inactivates the Ca^{2+}-ATPase.

Assays. The time and temperature of the incubations were chosen to fall within the range for which the assay gave linearity with time. Additional details are given in the legends to the figures. Except for the ATPase measurements on the reconstituted ATPase, all assays were performed in 0.5 ml of medium. The ATPase measurements on solubilized ATPase were measured on 1–5 μg of enzyme per tube in a medium which was 25 mM in Tes-triethanolamine, pH 7.4; no calmodulin was present.

The experiments on Ca^{2+} transport were done in a medium which was 9 mM in histidine-imidazole, pH 7.1, 0.6 mM in Tris-glycylglycine, 40 mM in NaCl and 7.5 mM in KCl. For the experiments shown in Fig. 1, Tris was substituted for histidine because of the high Mg^{2+} content of the histidine available. Ca^{2+} uptake by the erythrocyte membrane vesicles was measured utilizing 0.2–0.5 mg membrane protein per tube.

We developed a new method of separating the membrane vesicles from their incubation medium

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

in order to obtain usable results for conditions in which the uptake was low, particularly in the absence of Mg^{2+}. This method gave good reproducibility and very much lower blanks: 1 ml of cold 40 mM Tris-glycylglycine, pH 7.1, containing 5 mM EGTA, was added to 500 µl of assay medium. The suspension was centrifuged 20 min at 100000 × *g*, the pellet resuspended in 1 ml cold 40 mM Tris-glycylglycine (without EGTA) and centrifuged again in the same way. The resulting pellet was resuspended in water and counted using a liquid scintillation counter.

The concentration of the various free ionic species were calculated as previously described [7]. The association constants previously used [7] were used with the following significant changes: the logarithm of the association constant for the reaction

$$MgEDTA^{2-} + H^{+} \rightleftharpoons MgHEDTA^{-}$$

was 3.85 and not 2.8, as was erronously used before. This change has little effect at pH values above 6. It is also important to point out that this constant is valid for the reaction as written above, and not for $HEDTA^{3-} + Mg^{2+} \rightleftharpoons MgHEDTA^{-}$, as written incorrectly in Table I of Ref. 7. All of the association constants are written, where possible, as a protonation and not as a binding of a metal ion by a protonated species. This is in agreement with the usage in the most recent edition of our primary reference [10], but differs from that used in the older editions of this same reference.

The log of the association constant for CaATP was taken to be 4.7 instead of 3.6. This is a very significant change, and is justified as follows: the association constants for MgATP and CaATP are highly dependent on ionic strength; ATP binds these metal ions much more strongly at zero ionic strength than at 0.1 ionic strength [11,12]. Since good data exists for the ionic strength dependence of the association constant for MgATP [12] and since $\log K_a$ for CaATP at an ionic strength of 0.1 is 0.3 less than that of ATP [11], $\log K_a$ for CaATP was assumed to be 0.3 units below that of MgATP at all ionic strengths. The $\log K_a$ for MgATP was assumed to be 5.00, not 4.85 as previously [7]. The change in the association constant for MgATP was due to a change in the assumed ionic strength. This was chosen to be 50 mM, an intermediate value reasonably well suited to all the experimental conditions utilized here. In addition to these previously used constants the following logarithms of association constants were also needed. $HEGTA^{3-}$, 9.47; H_2EGTA^{2-}, 8.85; H_3EGTA^{-}, 2.66; H_4EGTA, 2.00; $CaEGTA^{2-}$, 10.97; $CaHEGTA^{-}$, 3.79; $MgEGTA^{2-}$, 5.21; $MgHEGTA^{-}$, 7.62.

It should be noted that the change in the value of the association constant used for CaATP gives rise to a change of more than one order of magnitude in the apparent CaATP concentration calculated. The actual value utilized for this association constant does not affect our reasoning, as we pointed out previously [7].

Results and Discussion

As mentioned above, the crucial experimental observation supporting the idea that free ATP is the substrate for this enzyme was the activity of the enzyme in the absence of Mg^{2+}. This activity has been confirmed in a number of laboratories [3,7] and is generally accepted. The activity is always lower than is seen in the presence of Mg^{2+}; it has been proposed that the higher rate of Ca^{2+}-ATPase in the presence of Mg^{2+} results from the stimulation by Mg^{2+} of the dephosphorylation of

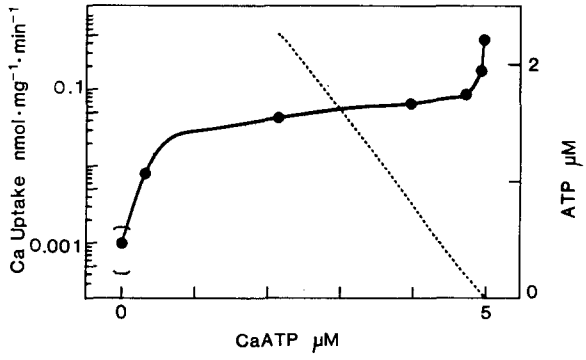

Fig. 1. Free ATP was not required for Ca^{2+} uptake. Total ATP was held constant at 5 µM, Mg^{2+} and ion chelators were absent, and Ca^{2+} was increased. The solid line and circles refer to the Ca^{2+} uptake, while the dotted line refers to the concentration of ATP^{4-}. Protonated forms of ATP without metal ions will be present in smaller amounts than ATP^{4-}. Total Ca^{2+} present was 0, 0.002, 0.021, 0.10, 0.50 2.5, and 10 mM, and 0.1 mM EGTA was present in the no Ca^{2+} sample. Incubation was for 30 min at 37°C.

the phosphoenzyme intermediate [3,13,14].

In the absence of Mg^{2+}, the enzyme must use either free ATP or CaATP as the substrate. Data indicating that CaATP is the substrate under these circumstances are shown in Fig. 1 of the present paper and in Fig. 5 of Ref. 7. In both of these experiments, enzyme activity was observed as a function of increasing Ca^{2+} at 1 μM total ATP. Under these circumstances CaATP and Ca^{2+} increased while free ATP was driven to extremely low levels. Fig. 1 shows this experiment for Ca^{2+} uptake by membrane vesicles, while Fig. 5 of Ref. 7 shows the same experiment for ATP hydrolysis by the purified enzyme. In both cases, the activity of the enzyme did not decrease with decreasing free ATP but rather continued to increase.

In a separate experiment, Ca^{2+} uptake was measured at increasing ATP concentrations in the absence of Mg^{2+}; the hypothetical K_m for free ATP was estimated from an Eadie-Hofstee plot to be 1.8 μM (data not shown). Since the free ATP concentration was reduced far below 1.8 μM, CaATP must be the substrate under these circumstances. The previous work on this subject assumed that free ATP was the substrate without critically examining the possibility that CaATP was the substrate in the absence of Mg^{2+}. The reason for this assumption was that free ATP is present at an appreciably higher concentration than CaATP under the most commonly used experimental conditions. The data do not rigorously exclude the possibility that free ATP might be utilized as a substrate in these experiments at CaATP concentrations below 2 μM. However, this possibility seems rather unlikely: CaATP is clearly the substrate at CaATP concentrations above 4 μM, so the enzyme would need to switch substrates from free ATP to CaATP as Ca^{2+} was increased. A site on the enzyme which has the high affinity for CaATP required to give the data in Fig. 1 would not be expected also to have a high affinity for a molecule with such a different charge and conformation as free ATP. The simplest explanation of all the data to date is that, in the absence of Mg^{2+}, CaATP is the substrate for this enzyme.

In the presence of Mg^{2+}, the situation is very different; even so, work on the purified Ca^{2+}-ATPase led me to the conclusion that CaATP was the substrate in this situation also [6,7]. Other workers have recently put forward the idea that MgATP is the substrate based on Ca^{2-} uptake data with vesicles [15,16]. The experiments reported here, taken together with those in Ref. 16 should give an idea of the current status of this matter.

A crucial observation, supporting the idea that CaATP is the substrate, was the inhibition of the purified ATPase by moderate concentrations of free Mg^{2+}; this inhibition was shown in Figs. 1 and 3 of Ref. 7. In the experiments of Ref. 7 half-maximal inhibition occurred at about 20 μM Mg^{2+} when the total ATP was 1 μM and 200 μM Mg^{2+} when the total ATP was 70 μM. At higher ATP and Ca^{2+}, Mg^{2+} concentrations up to 20 mM had no inhibitory effect. The observation that higher Mg^{2+} was required for inhibition at higher ATP is exactly what was expected from our model: If CaATP were the substrate, Mg^{2+} and Ca^{2+} would compete for the same site on ATP; replacement of Ca^{2+} by Mg^{2+} would inhibit the enzyme by depriving it of its substrate.

However, when EGTA, rather than EDTA, was used to buffer the Ca^{2+} concentration, the characteristics of the Mg^{2+} inhibition were strikingly changed. Fig. 2 shows the dependence of the ATPase of the purified enzyme on Mg^{2+} concentration at three different total ATP concentrations. The shape of the curve showing dependence of activity on free Mg^{2+} is independent of the total ATP concentration; inhibition to half the maximum activity occurs at about 10 mM Mg^{2+} at all three ATP concentrations. A similar experiment, giving similar results for Ca^{2+} uptake, is shown in Fig. 2 of Ref. 16. This may be contrasted with Fig. 3, which shows the predicted dependence of ATPase activity on Mg^{2+} if the Mg inhibition were caused by the displacement of Ca from its complex with ATP. The curves were calculated for the same three total ATP concentrations used for Fig. 2 and for Fig. 2 of Ref. 16. Equations 1–5 of Ref. 7 were used with the K_m for Mg^{2+} equal to 2 μM, the total enzyme equal to 1 mg and the other kinetic constants derived from Fig. 6 of Ref. 7. The important point to be drawn from Fig. 3 is that, if Mg^{2+} were inhibiting by displacing Ca^{2+} from ATP, the amount of Mg^{2+} required for half-maximal inhibition of the ATPase would shift

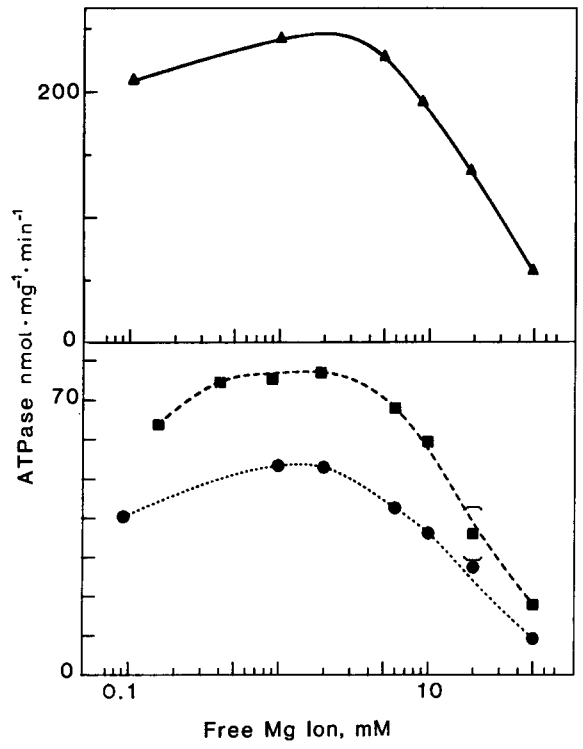

Fig. 2. Mg^{2+} and ATP concentration dependence of ATPase in the purified enzyme. The incubation medium was 25 mM in Tes-triethanolamine, pH 7.4, 150 mM in KCl, and 0.100 mM in EGTA. The total Ca^{2+} was varied slightly to maintain the free Ca^{2+} at 2.0 μM; the total Ca^{2+} varied from 0.108 mM (at 1 mM ATP and 1 mM total Mg^{2+}) to 0.090 mM (at 50 mM Mg^{2+}). The assay was done as a function of free Mg^{2+} at 10 μM ATP (circles), 100 μM ATP (squares) and 1 mM ATP (triangles). Incubation times were 10, 66 and 92 min, respectively, all at 37°C. ATPase concentration 5.6 μg/ml.

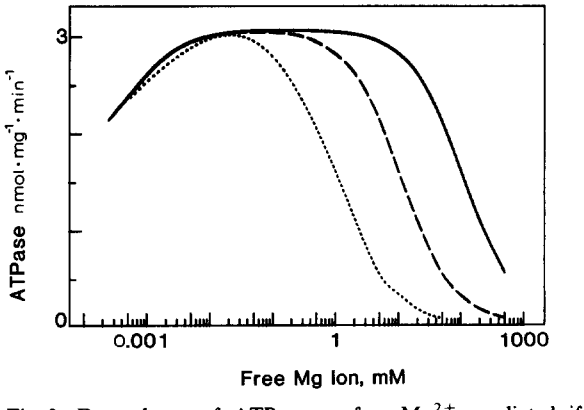

Fig. 3. Dependence of ATPase on free Mg^{2+} predicted if Mg^{2+} inhibition was caused by the competition of Mg^{2+} and Ca^{2+} for ATP. Calculations were done for the experimental conditions used in the experiment of Fig. 2; the lines refer to different total ATP concentrations as follows: Dotted line, 10 μM; dashed line, 100 μM; solid line, 1 mM.

in the manner shown in the figure; it is clear from Fig. 2 and from Fig. 2 of Ref. 16 that such a shift does not occur when CaEGTA is kept constant.

The considerations discussed above indicate that the inhibition of the ATPase by high Mg^{2+} concentrations is probably caused by competition of Mg^{2+} and Ca^{2+} for a site on the enzyme, and not for ATP. This conclusion significantly undercuts the argument previously made from this laboratory for CaATP as the substrate [6,7].

The situation is complicated by the effects of EGTA and EDTA on the enzyme's kinetics [17,18]. Some of the differences between the results reported here and those reported previously [7] may stem from the fact that EDTA was used as the metal ion buffer in the previous experiment, while EGTA is used in the experiments reported here. When EDTA was used, it was necessary to vary the total Ca^{2+} over a wide range in order to maintain a constant free Ca^{2+}. At high Mg^{2+}, the total Ca^{2+} became very low. The relatively easy inhibition of the reaction by Mg^{2+} may partly be related to this. When EGTA is used as a buffer, total Ca^{2+} remains almost constant over a wide range of Mg^{2+} concentrations. Although this appears to be a plausible explanation for the differences between the previous results and those reported here, attempts to demonstrate this conclusively were not successful.

The evidence so far put forward in favor of MgATP as the substrate of the enzyme is not conclusive. In general, it consists of demonstrations that CaATP can be reduced to a low concentration without inhibiting the enzyme (Fig. 6 of Ref. 16 and Fig. 2 of Ref. 15). However, the levels of CaATP achieved were not sufficiently low; an enzyme with the affinity for CaATP previously reported [6,7] would still be saturated with CaATP at the lowest concentration achieved.

With the techniques presently in use, it is difficult conclusively to demonstrate the nature of the substrate for the Ca^{2+}-ATPase from erythrocyte membranes. MgATP appears to be the most likely candidate at present, but more definitive experiments are required to arrive at the final answer to this question.

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References

1 Wolf, H.U. (1972) Biochim. Biophys. Acta 266, 361–375
2 Wolf, H.U., Dieckvoss, G. and Lichtner, R. (1977) Acta Biol. Med. Germ. 36, 847–858
3 Rega, A.F. and Garrahan, P.J. (1975) J. Membrane Biol. 22,313–327
4 Schatzmann, H.J. (1977) J. Membrane Biol. 35, 149–158
5 Sarkadi, B., MacIntyre, J.D. and Gárdos, G. (1978) FEBS Lett. 89, 78–82
6 Penniston, J.T., Graf, E., Niggli, V., Verma, A.K. and Carafoli, E. (1980) in Calcium-binding Proteins and Calcium Function (Siegel, F.L., Carafoli, E., Kretsinger, R.H., MacLennan, D.H. and Wasserman, R.H., eds.), pp. 23–30, Elsevier-North Holland, Amsterdam
7 Graf, E. and Penniston, J.T. (1981) J. Biol. Chem. 256, 1587–1592
8 Steck, T.L., Weinstein, R.S., Straus, J.H. and Wallach, D.F.H. (1970) Science 168, 255–257
9 Blostein, R. and Chu, L. (1977) J. Biol. Chem. 252, 3035–3043
10 Martell, A.E. and Smith, R.M. (1974) Critical Stability Constants, Vol. 1, Plenum Press, New York
11 Smith, R.M. and Martell, A.E. (1975) Critical Stability Constants, Vol. 2, Plenum Press, New York
12 Adolfsen, R. and Moudrianakis, E.N. (1978) J. Biol. Chem. 253, 4378–4379
13 Rega, A.F. and Garrahan, P.J. (1978) Biochim. Biophys. Acta 507, 182–184
14 Garrahan, P.J. and Rega, A.F. (1978) Biochim. Biophys. Acta 513, 59–65
15 Sarkadi, B., Enyedi, A. and Gárdos, G. (1981) Cell Calcium 2, 449–458
16 Enyedi, A., Sarkadi, B. and Gárdos, G. (1982) Biochim. Biophys. Acta 687, 109–112
17 Sarkadi, B., Schubert, A. and Gárdos, G. (1979) Experientia 35, 1045–1047
18 Al-Jobore, A. and Roufogalis, B.D. (1981) Biochim. Biophys. Acta 645, 1–9