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TRANSIENT STATE KINETIC STUDIES OF PHOSPHORYLATION BY ATP AND P_i OF THE CALCIUM-DEPENDENT ATPase FROM SARCOPLASMIC RETICULUM

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Summary

The ATPase of the sarcoplasmic reticulum is phosphorylated by ATP in the presence of Ca^{2+}. A rapid phosphorylation was observed when the enzyme was preincubated with Ca^{2+} prior to the addition of 0.1 or 1 mM ATP. The rate of phosphorylation was decreased when Ca^{2+} was omitted from the preincubation medium and added with ATP when the reaction was started. The rate of phosphorylation by ATP was further decreased when P_i was included in the preincubation medium without Ca^{2+}. In this case, the enzyme was phosphorylated by P_i during the preincubation. When Ca^{2+} and ATP were added, a burst of phosphorylation by ATP was observed in the initial 16 ms. In the subsequent incubation intervals, the phosphorylation by ATP was synchronous with the hydrolysis of the phosphoenzyme formed by P_i.

The rate of hydrolysis of the phosphoenzyme formed by P_i was measured when either the P_i concentration was decreased 10 fold, or when Ca^{2+}, ATP or ATP plus Ca^{2+} was added to the medium. Upon the single addition of Ca^{2+}, the time for half-maximal decay was in the range 500—1000 ms. In all other conditions it was in the range 70—90 ms.

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Introduction

The Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum is able to catalyse both the synthesis and hydrolysis of ATP. The activity of this enzyme is modulated by the binding of Ca^{2+} on the two sides of the membrane [1—3]. Hydrolysis of ATP is initiated by the transfer of the γ-phosphate of ATP to the membrane-bound enzyme forming an acylphosphate protein. This is activated by the binding of Ca^{2+} to a site of high affinity located on the outer surface of the vesicles membrane [1—3]. The phosphoprotein formed represents an intermediary product in the sequence of reactions leading to Ca^{2+} transport and P_i liberation. The synthesis of ATP is promoted by the reversal of the intermediary reactions responsible for the hydrolysis of ATP. Accordingly, the synthesis is initiated by the phosphorylation of the enzyme by P_i. This reaction is inhibited by the binding of Ca^{2+} to the same enzyme site which activates the phosphorylation by ATP [1,3—11]. On the basis of accumulated evidence the following reaction sequence was proposed [12]:

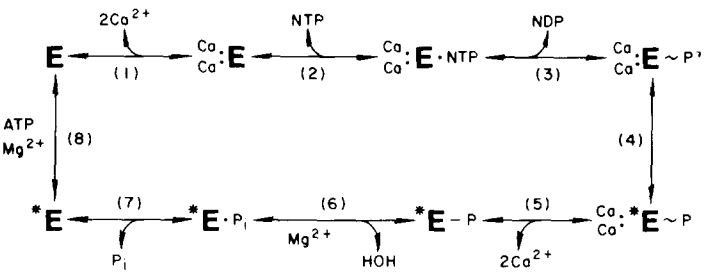

Fig. 1.

In this sequence, the Ca^{2+}-ATPase is represented in two different conformations, E and *E. In the E form, the site which translocates Ca^{2+} through the membrane face outward from the surface of the membrane and has a high affinity for Ca^{2+}. E can be phosphorylated by ATP but not by P_i. In the *E conformation, the Ca^{2+}-binding site faces the vesicle lumen and has a low affinity for Ca^{2+}. *E can be phosphorylated by P_i but not by ATP. In previous reports [12—14] steady-state kinetics evidences were presented indicating that the interconversion of *E into E (Reaction 8) is the slowest step of the reaction sequence and that ATP, besides phosphorylating the enzyme, can also activate the rate of interconversion of *E to E. In this report millisecond mixing and quenching experiments were performed in order to characterize further this regulatory step.

Methods

Previous publications describe methods for preparing sarcoplasmic reticulum vesicles [15], leaky vesicles, i.e. vesicles rendered permeable to Ca^{2+} [16], [γ-^{32}P]ATP [17], purification of $^{32}P_i$ [18], and measurement of the phosphoenzyme formation [8,12].

The mixing and quenching experiments were performed with a multispeed transmission device which force mixed together through a capillary the solu-

tions contained in two syringes [10]. One end of the capillary tube was connected to the syringes by means of a 'Y' junction. The other end was immersed in the quenching solution (4 mM P_i in 250 mM $HClO_4$). Except for the experiment of Fig. 4, the reaction was started by mixing equal volumes of the solution contained in the syringes. For the dilution experiments described in Fig. 4 the reaction was started by mixing one volume of syringe A with 10 vols. of syringe B. In this experiment, syringes with different capacities were used. Reaction times ranging from 16 to 654 ms were obtained by varying both the length of the capillary tube and the flow rate of injection. The reaction was performed at room temperature (22—25°C). The composition of the solutions contained in the syringes is specified in the figure legends. In the text, P_i refers to a NaH_2PO_4/Na_2HPO_4 buffer (pH 6.0). The maximum level of phosphoenzyme was found to vary significantly among the different vesicle preparations tested. Therefore, whenever possible, each set of experiment was performed with same vesicle preparation.

Results

Phosphorylation by ATP: preincubation with Ca^{2+}, EGTA or EGTA plus P_i

The aim of the experiments described in Fig. 2 was to measure the rate of phosphorylation of the vesicles by ATP starting from different points of reaction sequence proposed, namely $^{Ca}_{Ca}E$, *E and *E-P. The enzyme form $^{Ca}_{Ca}E$ is formed when leaky vesicles are incubated in presence of Ca^{2+} [3]. A rapid phosphorylation of the enzyme was observed when ATP was added to the reaction mixture to a final concentration of either 0.1 or 1 mM. In the time

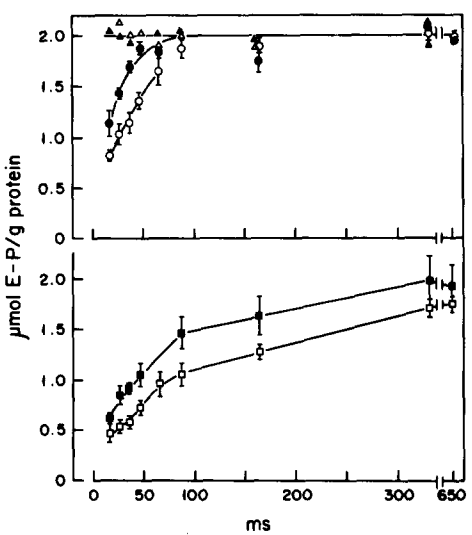

Fig. 2. Phosphorylation by ATP: Preincubation of the enzyme with either Ca^{2+}, EGTA, or EGTA plus P_i. Syringe A contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 1.0 mg/ml of leaky vesicles protein and either 0.1 mM $CaCl_2$ (△, ▲), 0.5 mM EGTA (○, ●) or 0.5 mM EGTA and 10 mM P_i (□, ■). Syringe B contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.2 mM (open symbols) or 2 mM (closed symbols) [γ-^{32}P]ATP and either 0.1 mM $CaCl_2$ (△, ▲) or 0.7 mM $CaCl_2$ (○, ●, □, ■). The values represent the average ±S.E. of four determinations.

scale resolution of the method used it was not possible to measure the initial rate of phosphorylation; the steady-state level was already reached within the initial 16 ms of reaction. This pattern did not vary when 5 mM P_i was added to the reaction mixture together with 0.1 mM ATP (data not shown). A slower rate of phosphorylation was measured when the vesicles were incubated with EGTA prior to the addition of ATP and Ca^{2+} to the medium. The rate of phosphorylation increased by raising the ATP concentration from 0.1 to 1 mM. When the steady state was reached, essentially the same level of phosphoenzyme was attained regardless of the ATP concentration used and on whether the vesicles were preincubated with Ca^{2+} or EGTA. These data indicate that when the enzyme is preincubated with EGTA, a slower step is included in the reaction sequence preceding the binding of ATP to the $^{Ca}_{Ca}E$ form (Reaction 2). In the reaction sequence proposed, the slower step is represented by the conversion of $^*E$ into $^{Ca}_{Ca}E$ (Reactions 8 and 1). During the preincubation with EGTA, the equilibrium reached between the form E and $^*E$ should be such as to favor the accumulation of $^*E$.

In previous reports [8—11,14,19] it was shown that the enzyme is phosphorylated by P_i when incubated with EGTA at pH 6.0. In the reaction sequence proposed the form phosphorylated by P_i is represented as $^*E$-P. Different rates of phosphorylation by ATP were observed when leaky vesicles were preincubated with EGTA (Fig. 2, top) or with EGTA plus P_i (Fig. 2, bottom). This was probably due to the conversion of $^*E$-P into $^*E$ (Reactions 7 and 6). Notice that in the presence of P_i, the rate of phosphorylation by ATP does not vary with the ATP concentration of the medium. Only the parcel of enzyme phosphorylated by ATP seems to vary during the initial 300 ms. Possible competition between P_i and ATP for $^{Ca}_{Ca}E$ cannot account for this finding since, as stated above, we failed to measure an effect of P_i on the rate of phosphorylation when the vesicles were preincubated with Ca^{2+}.

Interconversion of the enzyme forms phosphorylated by P_i and ATP

The amount of $^*E$-P formed when the vesicles are incubated with EGTA can be measured with the use of $^{32}P_i$ [8—11,14,19]. The experiment of Fig. 2 (bottom) indicates that the addition of ATP and Ca^{2+} to the medium promotes the cleavage of $^*E$-P in order to yield the enzyme form $^{Ca}_{Ca}E$ which is then phosphorylated by ATP. This was tested in Fig. 3. Two parallel experiments were performed; in one $^{32}P_i$ and non-radioactive ATP were used and in the second non-radioactive P_i and [γ-^{32}P]ATP were used. In the initial 16 ms a burst of phosphorylation by ATP was observed (0.63 μmol/g of protein). This was not accompanied by a significant decrease of the parcel of enzyme phosphorylated by $^{32}P_i$ (from 1.24 to 1.17 μmol/g of protein). In the subsequent incubation intervals the increment of the parcel of the enzyme phosphorylated by ATP was synchronous with the disappearance of the enzyme form phosphorylated by P_i. The additional parcels of enzyme phosphorylated by ATP between 16 and 46 ms and between 46 and 654 ms were, respectively, 0.41 and 0.86 μmol/g of protein while the disappearance of the phosphoenzyme formed by $^{32}P_i$ in these time intervals were, respectively, 0.36 and 0.69 μmol/g of protein (Fig. 3). Notice that the level of phosphoenzyme formed by P_i during the preincubation was 1.24 μmol/g of protein while when the steady state was reached after

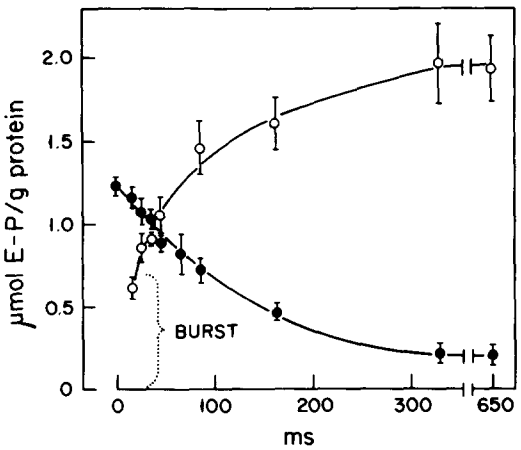

Fig. 3. Coupling between the phosphorylation by ATP and the cleavage of the phosphoenzyme formed by P_i. Syringe A contained 30 mM Tris/maleate buffer (pH 6.0), 0.5 mM EGTA, 10 mM $MgCl_2$, 10 mM P_i and 1 mg/ml of vesicle protein. Syringe B contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.7 mM $CaCl_2$ and 2 mM ATP. The values represent the average ±S.E. of six determinations. ○, non-radioactive P_i and [γ-^{32}P]ATP; ●, $^{32}P_i$ and non-radioactive ATP.

the addition of ATP and Ca^{2+} the total level of phosphoenzyme was 2.10 μmol/g of protein. This difference and the initial burst of phosphorylation by ATP indicates that during the preincubation with EGTA and P_i the equilibrium reached between the different enzyme forms (E, *E and *E-P) included a form which could readily interact with Ca^{2+} and ATP (E) but did not react with P_i. This enzyme form should bind and react with Ca^{2+} and ATP in less than 16 ms. According to this reasoning the binding of Ca^{2+} to E (Reaction 1) is much faster than the interconversion of *E into E (Reaction 8). Therefore the delay of phosphorylation by ATP in the presence of EGTA observed in Fig. 2 cannot be accounted by Reaction 1.

Hydrolysis of the phosphoenzyme formed by P_i

The experiments of Figs. 2 and 3 lead to the conclusion that Reactions 1—3 are faster than Reactions 6—8. The experiments of Figs. 4 and 5 were designed to distinguish which of the last three reactions is the rate-limiting step. The rate of phosphoenzyme hydrolysis in absence of Ca^{2+} (Reactions 6 and 7) can be measured by suddenly modifying the equilibrium between *E-P and *E. This can be attained either by adding ATP or by decreasing the P_i concentration of the medium. In previous reports [8,20] it was shown that in the absence of Ca^{2+}, ATP competitively inhibits the phosphorylation of the enzyme by P_i. In both conditions, the time for half-maximal hydrolysis of phosphoenzyme was found to be in the range 70—90 ms (Figs. 4 and 5). In agreement with previous reports [10,11], a slow rate of phosphoenzyme hydrolysis was observed upon the addition of Ca^{2+} to the incubation medium (Fig. 5) the time for half-maximal decay being in the range of 500—1000 ms. The dephosphorylation of the enzyme by Ca^{2+} is due to the accumulation of the enzyme form $^{Ca}_{Ca}E$ [8,10, 11,14,21]. From these data it is concluded that the rate of phosphoenzyme hydrolysis (Reactions 6 and 7) is faster than the rate of conversion of *E into

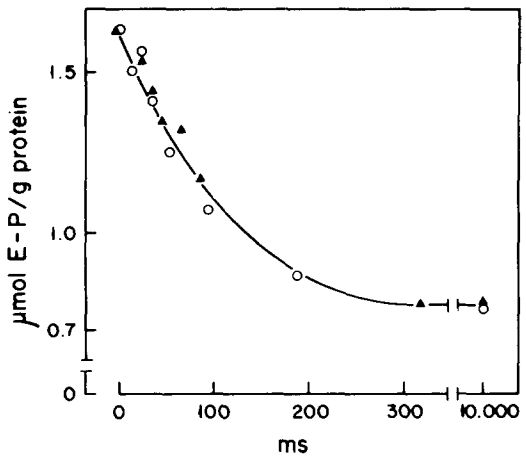

Fig. 4. Phosphoenzyme hydrolysis in the absence of Ca^{2+}. Dilution of $^{32}P_i$ from 5 to 0.42 mM (○): syringe A contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.5 mM EGTA, 5 mM $^{32}P_i$ and 1 mg/ml leaky vesicles protein. The solution contained in syringe B was the same as syringe A except that $^{32}P_i$ and vesicles were omitted. The reaction was started by mixing one volume of solution contained in syringe A with ten volumes of solution contained in syringe B. The values represented the average of two experiments. Addition of ATP (▲): Syringe A contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.5 mM EGTA, 10 mM $^{32}P_i$ and 1 mg/ml leaky vesicles protein. Syringe B contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$ and 2 mM ATP. The reaction was started by mixing equal volumes of syringes A and B. The values represented the average of four experiments.

$^{Ca}_{Ca}E$ (Reactions 8 and 1). Since Reaction 1 is completed in less than 16 ms (Fig. 3) it is concluded that Reaction 8 is the rate-limiting step of the reaction cycle. The rate of phosphoenzyme hydrolysis was accelerated when ATP was added simultaneously with Ca^{2+} to the reaction medium (Fig. 4), the time for half-maximal dephosphorylation being in the range of 70—90 ms. The findings with ATP (Figs. 3—5) can be interpreted by a possible competition between P_i and ATP in Reaction 7 leading to the formation of a dead-end complex *E-ATP

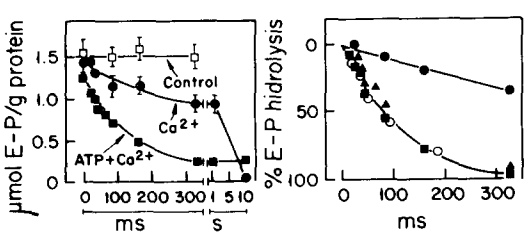

Fig. 5. Phosphoenzyme hydrolysis in the presence of Ca^{2+}. Left. Syringe A contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, 0.5 mM EGTA, 10 mM $^{32}P_i$ and 1 mg/ml of leaky vesicles protein. Syringe B contained 30 mM Tris/maleate buffer (pH 6.0), 10 mM $MgCl_2$, and either (●) 0.7 mM $CaCl_2$ or (■) 0.7 mM $CaCl_2$ plus 2 mM ATP. In previous reports [3,8] it was shown that at pH 6.0 the apparent K_m for the phosphorylation of the enzyme by P_i is in the range of 1—2 mM; □, a control experiment which shows that in the experimental conditions used, there was no detectable variation of the phosphoenzyme level upon dilution of the P_i concentration from 10 to 5 mM. In this experiment, the solution contained in syringe A was the same as described above. Syringe B contained the same solution as syringe A except that $^{32}P_i$ and vesicles were omitted. The values represent the average ±S.E. of six determinations. Right. The percent of the parcel of phosphoenzyme hydrolyzed in the experiments of left (●, ■) and Fig. 4 (○, ▲) were plotted as a function of the reaction time. ●, addition of 0.2 mM Ca^{2+}; ■, 0.2 mM Ca^{2+} plus 2 mM ATP; ○, P_i dilution from 5 to 0.46 mM; ▲, 2 mM ATP in the absence of Ca^{2+}.

which would not react with either P_i or low Ca^{2+} concentrations. Alternatively, the binding of ATP to the enzyme would convert the form *E into another enzyme form which can be phosphorylated by ATP in the presence of Ca^{2+}. The data of Figs. 2 and 3 favor the second hypothesis. In presence of Ca^{2+}, after the initial 16 ms of reaction, the cleavage of *E-P is accompanied by the formation of $^{Ca}_{Ca}$E-P (Fig. 3). When the steady state is reached, the amount of phosphoenzyme does not decrease by increasing the concentration of ATP (Fig. 2) as it should be expected in the case of the formation of the inactive complex *E-ATP. These data support the conclusion that the complex *E-ATP is readily converted into E-ATP, thus liberating the rate-limiting step of the reaction sequence. At present we do not know whether the catalytic site (Reaction 3) and the regulatory site (Reaction 8) of ATP are the same.

Discussion

In the studies reported here, leaky vesicles were used in order to avoid the variation of the Ca^{2+} concentration in the vesicles lumen during the different intervals of reaction. In previous reports [12—14,22] it was shown that the steady-state level of phosphoenzyme varies depending on the binding of Ca^{2+} to a site of low affinity located in the inner surface of the vesicles membrane (Reaction 5). The affinity of the Ca^{2+}-dependent ATPase for P_i varies with the pH of the medium, being maximal at pH 6.0 [8—11,14]. Therefore, all the experiments were performed at this pH.

The apparent K_m of ATP for the enzyme phosphorylation in presence of Ca^{2+} is in the range of 5—20 μM [23,24]. On the other hand, the ATP concentration dependence of ATP hydrolysis is complex and cannot be fitted with a single straight line in a double-reciprocal plot [25—30]. A further activation of ATP hydrolysis is observed in the ATP concentrations above 0.1 mM, i.e. in concentrations above those required for maximal phosphorylation of the enzyme. This has been attributed to an activating effect of ATP on an intermediary step of the reaction cycle. Different mechanisms have been proposed to account for this regulatory effect of ATP. Kanazawa and coworkers [2,27] suggested that ATP can form two types of Michelis complex with the enzyme, E_1-ATP and E_2-ATP and proposed that the rate of conversion of the first complex into the second is accelerated by high concentrations of ATP. Alternatively, evidences have been presented that high concentrations of ATP accelerates the rate of phosphoenzyme hydrolysis [12—14,29,31,32]. The data presented in this report favor the second hypothesis (Figs. 3 and 4). ATP at high concentrations binds to *E, accelerating its conversion into a form which can be phosphorylated by ATP in presence of Ca^{2+}. The draining of the form *E would accelerate the hydrolysis of *E-P by displacing the equilibrium of Reactions 6 and 7 towards left. This conclusion is further supported by steady-state kinetics data previously reported. Coupled with the phosphorylation by P_i, the enzyme catalyzes a rapid oxygen exchange between P_i and water [14,18,19]. The $P_i \rightleftharpoons HOH$ exchange is the result of Reactions 7 and 6 flowing forward and backwards. Both the phosphorylation by P_i and the $P_i \rightleftharpoons HOH$ exchange are impaired by the binding of Ca^{2+} to the site of high affinity. This inhibition is overcome by the addition of ATP to the medium. In steady-state

conditions the enzyme is simultaneously phosphorylated by P_i and ATP. The parcel of enzyme phosphorylated by P_i [12–14,21] and the rate of $P_i \rightleftharpoons HOH$ exchange [14] are smaller when higher the ATP concentration in the medium. These data indicate that the cycle of ATP hydrolysis includes the formation of *E and that in the steady state, the amount of *E available depends on the ATP concentration in the medium.

In the reaction sequence it is proposed that the enzyme undergoes a conformational change in Reaction 8. Recently, Dupont [33] and Dupont and Leigh [34] have shown that the binding of Ca^{2+} to the high affinity site results in an increase of the intrinsic fluorescence of the enzyme, which is reversed by the addition of EGTA to the medium.

Finally, while this report was in preparation, Rauch et al. [35] reported an experiment similar to that described in Fig. 2 (top). These authors did not study the combined effects of ATP and P_i on the rate of enzyme phosphorylation. They also concluded that when the enzyme is preincubated with EGTA, a slow reaction preceedes the phosphorylation by ATP. On the grounds of the data reported, Rauch et al. [35] proposed that the rapid binding of Ca^{2+} to the enzyme form a complex E_1-Ca^{2+} which is then slowly converted into a different E_2-Ca^{2+} complex capable of being phosphorylated by ATP. A similar mechanism has been proposed by Dupont and Leigh [34].

Acknowledgments

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