Substrate Regulation of the Sarcoplasmic Reticulum ATPase

TRANSIENT KINETIC STUDIES*

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The rate of phosphorylation of the Ca²⁺-dependent ATPase of sarcoplasmic reticulum vesicles by ITP and ATP was studied using a millisecond mixing and quenching device. The rate of phosphorylation was slower when the vesicles were preincubated in a Ca²⁺-free medium than when preincubated with Ca²⁺, regardless of the substrate used and of the pH of the medium. When the vesicles were preincubated with Ca²⁺ at pH 7.4 an overshoot of phosphorylation was observed in the presence of ITP. The overshoot was abolished when the pH of the medium was decreased to 6.0 or when the vesicles were preincubated in a Ca²⁺-free medium. Using vesicles preincubated with Ca²⁺ the apparent $K_m$ for ITP found was 2.5 mM at pH 6.0 and 1.0 mM at pH 7.4. The $V_{\text{max}}$ observed (77 μmol g⁻¹ s⁻¹) did not change with the pH of the medium. Both at pH 6.0 and 7.4 the apparent $K_m$ for ATP was 3 μM when the vesicles were preincubated with Ca²⁺ and 50 μM when preincubated in a Ca²⁺-free medium. At pH 6.0 the $V_{\text{max}}$ for ATP varied from 96 to 33 μmol g⁻¹ s⁻¹ depending on whether the vesicles were preincubated in the presence or absence of Ca²⁺. At pH 7.4 the $V_{\text{max}}$ for ATP was 90 μmol g⁻¹ s⁻¹ in both conditions. The rate of phosphorylation of the vesicles was dependent on the relative Ca²⁺ and Mg²⁺ concentrations of the reaction medium regardless of the substrate used.

The mechanism of this regulatory role of ATP is still controversial (4, 13–16). The apparent affinity of the enzyme for ITP is more than 1 order of magnitude lower than that for ATP (4). Thus in the concentration range of 0.1 to 0.5 mM ATP acts both as a substrate and as an activator while ITP operates only as a substrate.

The aim of this report is to study the mechanism by which ATP regulates the activity of the Ca²⁺ transport ATPase. For this purpose the rates of the enzyme phosphorylation by either ATP or ITP were measured with the use of a millisecond mixing and quenching device.

MATERIALS AND METHODS

Previous publications describe methods for preparing sarcoplasmic reticulum vesicles (17), leaky vesicles (18), $[\gamma$-³²P]ATP (19), $[\gamma$-³²P]-ITP (19), and measurement of the phosphoryne enzyme formation (20, 21). Leaky vesicles were used in order to avoid the variation of the Ca²⁺ concentration in the vesicles lumen during the different intervals of reaction.

Quenched flow measurements were performed using a multiperfusion transmission device model 600.000 (Harvard Apparatus, Co., Inc., Dover, Mass.) as previously described (22). Equal volumes of solutions contained in two syringes were forced mixed together through a capillary tube connected by means of a "Y" junction. The other end of the capillary was immersed in the quenching solution (4 mM P, in a 250 mM perchloric acid solution). Different reaction times were obtained by varying both the length of the capillary tube and the flow rate of injection.

The reactions were performed at room temperature (22–25°C). The composition of the solutions contained in the syringes is specified in the figure legends. All the reagents used were of analytical grade.

RESULTS

Phosphorylation by ITP—Different rates of phosphorylation were obtained depending on whether the vesicles were preincubated with Ca²⁺ or EGTA. At pH 7.4, when the vesicles were preincubated with Ca²⁺, the addition of ITP (0.5 mM) resulted in a rapid phosphorylation of the enzyme during the initial 50 to 70 ms (Fig. 1, left). This was followed by a decrease of the phosphoryne enzyme level until the steady state was reached. A slower rate of phosphorylation was observed when vesicles were preincubated with EGTA prior to the addition of ITP and Ca²⁺. In this condition the overshoot of phosphorylation was abolished and the level of phosphoryne enzyme attain in the steady state was essentially the same as that obtained with vesicles preincubated with Ca²⁺. At pH 6.0 the rate of phosphorylation obtained with vesicles preincubated with Ca²⁺ was also faster than the rate measured with vesicles preincubated with EGTA (Fig. 1, right). However, at pH 6.0, both rates were slower than those measured at pH 7.4, the overshoot was abolished and the steady state level of phosphoryne enzyme decreased.

Using vesicles preincubated with Ca²⁺ the velocities of...
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**FIG. 1 (left). Phosphorylation by ITP.** Preincubation of the enzyme with Ca²⁺ or with EGTA. Left, Syringe A contained 1.0 mg/ml of leaky vesicles, 30 mM Tris/maleate buffer (pH 7.4) and either 0.1 mM CaCl₂ (●) or 0.2 mM EGTA (▲). Syringe B contained 30 mM Tris/maleate buffer (pH 7.4), 10 mM MgCl₂, and either 1.0 mM [γ-³²P]ITP (●) or 1.0 mM [γ-³²P]ITP plus 0.3 mM CaCl₂ (▲). Right, same experimental conditions and symbols of left except that the pH of the media was 6.0 and the CaCl₂ concentration used were 0.2 mM (●) or 0.4 mM (▲). Other experimental conditions were as described under “Materials and Methods.” The values represent the average ± S.E. of four experiments.

**FIG. 2 (right).** Velocities of phosphorylation as a function of ITP concentrations. Syringe A contained 1.0 mg/ml of leaky vesicles and either 30 mM Tris/maleate buffer (pH 7.4) plus 0.2 mM CaCl₂ (●) or 30 mM Tris/maleate buffer (pH 7.4) plus 0.1 mM CaCl₂ (▲). Syringe B contained 10 mM MgCl₂, 30 mM Tris/maleate buffer, pH 6.0 (●) or pH 7.4 (▲), and [γ-³²P]ITP concentrations as indicated in the figure. The reaction time was 26 ms. The values represent the average ± S.E. of four experiments.

**FIG. 3 (left). Phosphorylation by ATP 5 μM.** Preincubation of the enzyme with Ca²⁺ or with EGTA. Left, Syringe A contained 0.050 mg/ml of leaky vesicles, and either 30 mM Tris/maleate buffer (pH 6.0) plus 0.2 mM CaCl₂ (●) or 30 mM Tris/maleate buffer (pH 7.4) plus 0.1 mM CaCl₂ (▲). Syringe B contained 0.01 mM [γ-³²P]ATP, 10 mM MgCl₂, and 30 mM Tris/maleate buffer, pH 6.0 (●) or pH 7.4 (▲). Right, same experimental conditions as left except that Syringe A contained 0.2 mM EGTA instead of CaCl₂ and Syringe B contained in addition either 0.4 mM CaCl₂ (●) or 0.3 mM CaCl₂ (▲). Other experimental conditions were as described under “Materials and Methods.” The values represent the average ± S.E. of six experiments.

**FIG. 4 (right). Phosphorylation by ATP 0.5 mM.** Preincubation of the enzyme with Ca²⁺ or with EGTA. Syringe A contained 1.0 mg/ml of leaky vesicles and 30 mM Tris/maleate buffer, pH 6.0 (open symbols) or pH 7.4 (closed symbols) with either 0.2 mM CaCl₂ (△, ◯), or 0.2 mM EGTA (●, ▲). Syringe B contained 1.0 mM [γ-³²P]ATP, 10 mM MgCl₂, and 30 mM Tris/maleate buffer, pH 6.0 (open symbols) or pH 7.4 (closed symbols) with 0.4 mM CaCl₂ (△, ◯), or no added CaCl₂ (△, ◯). The values represent the average ± S.E. of four experiments.

Phosphorylation as a function of the ITP concentration was measured in Fig. 2. This experiments shows that although the Vₘₐₓ of phosphorylation does not vary with the pH of the medium (77 μmol g⁻¹ s⁻¹), the apparent Kₘ of the enzyme of ITP is higher at pH 6.0 (2.5 mM) than at pH 7.4 (1.0 mM). This can account for the different rates of phosphorylation observed in Fig. 1 with vesicles preincubated with Ca²⁺ in terms of the amount of Michaelis complex E-ITP formed.

Phosphorylation by ATP—For the experiments of Figs. 3 and 4, two different ATP concentrations were used. When the vesicles were preincubated with Ca²⁺, the addition of 5 μM ATP (Fig. 3) resulted in a rapid phosphorylation of the enzyme during the initial 100 ms. Essentially the same profile of enzyme phosphorylation were obtained at pH 6.0 and 7.4. The level of phosphoenzyme seems to decrease a little in the incubation intervals ranging between 200 and 300 ms. This
part of the curve could not be clearly resolved due to the scattering of the experimental values obtained. Incubation intervals above 330 ms were avoided due to the low ATP concentration used and to the lack of an ATP regenerative system in the assay medium. A slow rate of phosphorylation was obtained when the vesicles were preincubated with EGTA (Fig. 3, right). In this case the rate of phosphorylation did vary with the pH of the medium, being slower at pH 6.0 than at pH 7.4.

When the ATP concentration in the medium was raised to 0.5 mM (Fig. 4) the initial rate of phosphorylation for vesicles preincubated with Ca"+ was too fast and could no longer be measured. When the vesicles were preincubated with EGTA the initial rate of phosphorylation could still be measured being faster at pH 7.4 than at pH 6.0. In contrast to Fig. 1, using 0.5 mM ATP, the steady state level of phosphoenzyme did not vary with the pH of the assay medium. In order to estimate the apparent Km for ATP and Vmax of phosphorylation, the amount of phosphoenzyme formed at 16 ms was measured as a function of ATP concentration (Fig. 5). For each given ATP concentration used the level of phosphoenzyme at 16 ms represents 30 to 66% of the maximal level obtained after 80 to 200 ms of reaction. When the vesicles were preincubated with Ca** the apparent Km and the Vmax of phosphorylation were 3 pM and 90 pMol g-1 s-1, respectively, and did not vary with the pH of the medium. Taking into account the Vmax of phosphorylation and the maximal level of phosphoenzyme obtained, the first order rate constant calculated is in the range of 36 to 45 s-1. With the use of intact vesicles and similar experimental conditions the rate constant of phosphorylation reported in the bibliography are 59 s-1 (16) and 85 s-1 (12). With the use of vesicles preincubated with EGTA, the apparent Km obtained was 50 pM both at pH 6.0 and 7.4. The Vmax of phosphorylation increased from 33 to 96 pMol g-1 s-1 when the pH of the medium was raised from 6.0 to 7.4.

With the use of leaky vesicles in the presence of 5 pM ATP the maximal level of phosphoenzyme obtained was in the range of 2.0 to 2.5 pMol/g of protein (Fig. 2) and did not vary when the ATP concentration was raised to 500 pM (Fig. 4). Contrasting with this finding, when intact vesicles were used, the maximal level of phosphoenzyme increased from 2.0 to 3.5 pMol/g of protein when the ATP concentration was raised from 5 to 500 pM (data not shown). This apparent discrepancy is probably related to the fact that only the intact vesicles are capable of accumulate Ca**. In a previous report (23) it has been shown that the steady state level of phosphoenzyme varies significantly depending on the substrate used and on whether or not the vesicles accumulate Ca**. With the use of intact vesicles, Froehlich and Taylor (16) and Verjovski-Almeida et al. (12) observed that the maximal level of phosphoenzyme obtained is in the range of 36 to 45 s-1. In Fig. 3 the enzyme was preincubated with Ca** and the rate of phosphorylation was calculated as a function of ATP concentration (Fig. 5). For each given ATP concentration used the level of phosphoenzyme at 16 ms represents 30 to 66% of the maximal level obtained after 80 to 200 ms of reaction. When the vesicles were preincubated with Ca** the apparent Km and the Vmax of phosphorylation were 3 pM and 90 pMol g-1 s-1, respectively, and did not vary with the pH of the medium. Taking into account the Vmax of phosphorylation and the maximal level of phosphoenzyme obtained, the first order rate constant calculated is in the range of 36 to 45 s-1. With the use of intact vesicles and similar experimental conditions the rate constant of phosphorylation reported in the bibliography are 59 s-1 (16) and 85 s-1 (12). With the use of vesicles preincubated with EGTA, the apparent Km obtained was 50 pM both at pH 6.0 and 7.4. The Vmax of phosphorylation increased from 33 to 96 pMol g-1 s-1 when the pH of the medium was raised from 6.0 to 7.4.

Effect of Ca** and Mg**—Kinetic studies of ATP hydrolysis indicated that the true substrate of the sarcoplasmic reticulum NTPase is the complex ATP-Mg (5). On the other hand, steady state level data show that the enzyme can be phosphorylated by ATP in the presence of Ca** and no added Mg** (23, 24). In these conditions, the steady state level of phosphoenzyme obtained is smaller than that obtained in presence of an optimal Mg** concentration. In Fig. 6 the enzyme was preincubated with Ca** and the rate of phosphorylation was measured in the presence of different Ca** and Mg** concentrations. In presence of 50 pM Ca** and no added Mg**, a slow rate of phosphorylation was measured. Upon the addition of 5 mM Mg**, the rate of phosphorylation was sharply increased and could no longer be measured with the method used. By raising the Ca** concentration to 30 mM in the presence of 5 mM Mg**, a slow initial rate of phosphorylation was detected, the steady state level of phosphoenzyme attained being the same as that measured in presence of 5 mM Mg** and 50 pM Ca**. The Km of the complex ATP-Mg and ATP-Ca are of the same order of magnitude (5). Therefore, in presence of 30 mM Ca** and 5 mM Mg**, the predominant species is ATP-Ca. These data agree with the conclusion that the complex ATP-Mg is the true substrate of the sarcoplasmic reticulum NTPase. However, do not exclude the possibility that the binding of Mg** to the enzyme and (or) the binding of Ca** to the low affinity site of the enzyme are involved in the regulation of the rate of phosphorylation. Froehlich (25) obtained reduced rates of enzyme phosphorylation by preincubating the enzyme in the presence of EGTA and increasing concentrations of Mg**. The author suggested that magnesium can bind to the free enzyme dislocating the equilibrium to an enzyme form that is not rapidly phosphorylated by ATP.

In Fig. 7 the enzyme was preincubated with Ca** and the rate of phosphorylation was measured in presence of 30 mM CaCl2 and 0.5 mM ITP at pH 7.4. In these conditions the rate of phosphorylation was decreased, the overshoot was abolished and the steady state level of phosphoenzyme increased reaching 5 pMol/g of protein (compare Fig. 1, left, and Fig. 7). The same reasoning proposed for Fig. 6 can also be applied for the decrease of the rate of phosphorylation observed in
The use of ATP or ITP in presence of 30 steady state level of phosphoenzyme were obtained with the state level of phosphoenzyme observed in Fig. 7. Different do not know the meaning of these findings. Perhaps this is activity.

Ca but not ITP. Ca is a powerful inhibitor of the NTPase 21, 26, 27). This could account for the increase of the steady low affinity Ca" binding site of the enzyme is saturated (15, 21, 26, 27). This could account for the increase of the steady state level of phosphoenzyme observed in Fig. 7. Different steady state level of phosphoenzyme were obtained with the use of ATP or ITP in presence of 30 mm Ca2+. At present we do not know the meaning of these findings. Perhaps this is related with the finding of Vianna (5) that the species ATP·Ca but not ITP·Ca is a powerful inhibitor of the NTPase activity.

**DISCUSSION**

The data presented in this report can be interpreted according to the following reaction sequence (24).

In this sequence, the enzyme is represented in two different conformations, \( E \) and \( *E \). In the \( E \) form, the site which translocates Ca++ through the membrane faces outward from the surface of the membrane and has a high affinity for Ca++ \( (K_i \approx 10^{-9} M) \). In the \( *E \) form the Ca++ binding site faces the inner surface of the vesicles membrane and has a low affinity for Ca++ \( (K_i \approx 10^{-2} M) \). The form \( *E \) is phosphorylated by ATP but not by Pi, while the form \( E \) is phosphorylated by Pi, but not by ATP. The conversion of \( *E \) into \( E \) (Reaction 8) is the slowest step of the reaction sequence. According to this sequence when the vesicles are preincubated with Ca++, the equilibrium reached between the different dephosphoenzyme forms would favor the accumulation of the form \( *E \) while in the presence of EGTA part of the enzyme is in the form \( *E \) (28). In all the conditions tested (Figs. 1, 3, and 4) the rate of phosphorylation was faster when the reaction was started with the enzyme in the form \( *E \). This clearly shows that when the vesicles are preincubated with EGTA, a slow reaction precedes the phosphorylation. In the reaction sequence proposed this slower step is represented by the conversion of \( *E \) into \( E \) (Reaction 8). A comparison of Figs. 3 to 5 indicates that this slow step is accelerated by ATP concentrations higher than those required for maximal phosphorylation of the enzyme. Two different apparent \( K_a \) for ATP were obtained depending on the preincubation conditions used. One of them \( (3.0 \mu M) \) reflects the binding of ATP to the catalytic site of the enzyme form \( *E \). The other \( K_a \) \( (50 \mu M) \) probably reflects the binding of ATP to the enzyme site involved in the regulation of the rate of conversion of \( *E \) into \( E \) (Reaction 8). If ATP had no effect in this step, essentially the same \( K_a \) for ATP should be obtained regardless of the preincubation conditions.

Figs. 2 and 5 show that when the vesicles are preincubated with Ca++, the \( V_{max} \) of phosphorylation does not vary with the pH of the medium regardless of the use of either ATP or ITP as substrate. This indicates that the pH of the medium has no effect after the formation of the complex \( *E \)-NTP. On the other hand different \( V_{max} \) of phosphorylation by ATP were obtained when the vesicles were preincubated with EGTA. In these conditions the Steps 8, 1, and 2 are included in the reaction sequence. At present we cannot discriminate whether the pH modifies the equilibrium of the forms \( E \) and \( *E \) during the preincubation with EGTA or whether modifies the effect promoted by ATP in this conversion.

With the use of ATP as substrate, Froehlich and Taylor (16, 29) observed an overshoot of phosphoenzyme. In our experimental conditions we were able to clearly detect an overshoot only with the use of ITP at pH 7.4 (Fig. 1). In the reaction cycle proposed the raising phase of phosphorylation could be related to the rapid phosphorylation of the enzyme form \( *E \) (Reaction 2) and the subsequent decay of the phosphoenzyme level do to the accumulation of the ITP unreactive enzyme form \( *E \). Thus the size of the overshoot would be greater when faster the rate of phosphorylation \( *E \) and slower the rate of conversion of \( *E \) into \( E \) (Reaction 8). Accordingly the overshoot was abolished when the rate of phosphorylation was decreased by lowering the pH of the medium from 7.4 to 6.0 (Fig. 1) and when the enzyme was preincubated with EGTA (Figs. 1, 3, and 4). The overshoot...
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also tends to disappear when 5 μM ATP was used as substrate (Fig. 3). This can indicate that the rate of conversion of $E_\text{calcium}$ into $E$ is already slightly accelerated in this ATP concentration. Notice that the second $K_m$ for ATP is 50 μM.

While this manuscript was in preparation Rauch et al. (30) and Sumida et al. (31) reported experiments measuring different rates of phosphorylation depending on whether the enzyme was preincubated in the presence of Ca$^{2+}$ or EGTA. These authors did not explore the effects of different NTP species, different ATP concentrations and of the pH of the medium on the delay of the rate of phosphorylation observed when the vesicles were preincubated with EGTA. On the ground of the data reported, Rauch et al. proposed that the binding of Ca$^{2+}$ to the enzyme forms a complex $E$-calcium. This would be followed by a slow isomerization process yielding a different $E$-calcium complex which is phosphorylated by ATP. A similar mechanism has been proposed by Dupont and Leigh (32). Alternatively Froelich proposed that the enzyme can exist in two different forms. Ca$^{2+}$ can bind to both of them. Only one of the forms would be phosphorylated by ATP and the rate limiting step of the catalytic cycle would be the isomerization of the ATP unreactive form. Both the free enzyme and the enzyme-calcium complex could isomerize yielding the enzyme form which is phosphorylated by ATP.

In conclusion there seems to be a general agreement that the sarcoplasmic reticulum NTPase can exist in two different forms only one of them being phosphorylated by NTP and that the conversion of one enzyme form into the other is a slow step which significantly influences the overall enzyme turnover. The data presented in this report suggest that ATP at high concentrations can accelerate the rate of this interconversion.

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