Mechanism of the Stimulation of Ca²⁺-dependent ATPase of Cardiac Sarcoplasmic Reticulum by Adenosine 3':5'-Monophosphate-dependent Protein Kinase

ROLE OF THE 22,000-DALTON PROTEIN*

(Received for publication, June 2, 1978)

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The effects of a cAMP-dependent protein kinase on the elementary steps of Ca²⁺-dependent ATPase of canine cardiac sarcoplasmic reticulum were examined in order to define the previously proposed regulatory role of the 22,000-dalton protein phospholamban. Cardiac sarcoplasmic reticulum, preincubated with cAMP, cAMP-dependent protein kinase, and unlabeled ATP in the presence and absence of MgCl₂, was assayed for the ATPase activity (Pₐ) and ADP liberation and formation of the intermediate of ATPase (EP) in media containing [γ-³²P]ATP and ionized Ca²⁺. Sarcoplasmic reticulum preincubated with MgCl₂ exhibited significant phosphorylation of phospholamban, whereas virtually no phosphorylation of this protein was seen in sarcoplasmic reticulum, determined by measuring PM, to 1 mM ATP, the rate of ATP hydrolysis by phosphorylated sarcoplasmic reticulum was markedly higher than that by control (unphosphorylated) sarcoplasmic reticulum. The effect was more evident at higher ATP concentrations, where the maximal velocity was approximately doubled. While EP levels of phosphorylated sarcoplasmic reticulum were almost equal to those of control sarcoplasmic reticulum at saturating concentration of Ca²⁺ (≥10 μM), phosphorylated sarcoplasmic reticulum exhibited pronounced reduction in EP levels at lower Ca²⁺ (0.1 to 10 μM). Thus, the ratio v/[EP], i.e. the rate of Pᵢ liberation per unit of concentration of EP, was more than 2-fold higher in phosphorylated sarcoplasmic reticulum than in control, suggesting that the rate of EP decomposition is markedly enhanced upon phosphorylation of phospholamban. The rate of EP decomposition of phosphorylated sarcoplasmic reticulum, determined by measuring its first order rate constant, kₑ, was approximately doubled, compared with control. These findings indicate that cAMP-dependent protein kinase-catalyzed phosphorylation of phospholamban causes an increase in the Ca²⁺-dependent ATPase activity through enhancement of the rate of EP decomposition and are in accord with the view that phospholamban can serve as a regulator controlling the active calcium transport by cardiac sarcoplasmic reticulum.

A brief incubation of cardiac microsomes, which consist mainly of sarcoplasmic reticulum, with cAMP-dependent protein kinase (EC 2.7.1.37, ATP:protein phosphotransferase) was shown to result in marked stimulation of Ca²⁺-dependent ATPase (EC 3.6.1.3, ATP phosphohydrolase) and calcium uptake (Tada et al., 1974; Kirchberger et al., 1974). This stimulation was subsequently found to be associated with cAMP-dependent protein kinase-catalyzed phosphorylation of a 22,000-dalton microsomal protein (Tada et al., 1975a). Based on these findings, a regulatory mechanism of calcium transport was proposed in which protein kinase-catalyzed phosphorylation of the 22,000-dalton protein, referred to as phospholamban (Tada et al., 1973, 1975a), serves as a modulator of Ca²⁺-dependent ATPase of cardiac sarcoplasmic reticulum (Katz et al., 1975, Tada and Kirchberger, 1975, Tada et al., 1975a, 1975b). This proposal was consistent with findings by a number of investigators who reported that phosphorylation of a microsomal protein of 20,000 to 22,000 daltons catalyzed by endogenous (LaRai and Morkin, 1974; Wray and Gray, 1977) and exogenous (Kirchberger and Chu, 1976; Schwartz et al., 1976; Tada et al., 1977b; Will et al., 1976) protein kinase can augment the rates of active calcium transport and Ca²⁺-dependent ATP hydrolysis by cardiac microsomes. While these studies pointed to the possibility that phospholamban may function as a regulatory component of Ca²⁺-dependent ATPase, none of these studies presented the direct evidence to explain, at the molecular level, the mechanism by which Ca²⁺-dependent ATPase is controlled by cardiac microsomes. It is well documented that in the active calcium transport by sarcoplasmic reticulum from skeletal and cardiac muscles, the ATPase enzyme of about 100,000 daltons serves as an energy transducer as well as a translocator of calcium ions (MacLennan and Holland, 1975; Tada et al., 1978b). In this communication, therefore, we sought to investigate whether protein kinase-catalyzed phosphorylation of phospholamban alters any one or more of the elementary steps of the enzymatic reactions of cardiac microsomal ATPase. It was found that phosphorylation of phospholamban causes a marked increase in the rate of decomposition of the phosphorylated protein kinase, adenosine 3’:5’-monophosphate-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N’-tetraacetic acid.
intermediate (EP) of the ATPase, resulting in increased turnover of the ATPase reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cardiac microsomes, which consist largely of fragmented sarcoplasmic reticulum, were prepared from dog heart ventricle by the procedures described previously (Harigaya and Schwartz, 1969; Kirchberger et al., 1974). Microsomes were stored on ice and used within several hours after preparation, unless otherwise indicated. In some cases, part of the microsomal preparation was frozen at −20°C in the presence of 0.5 M sucrose, 50 mM KCl, and 20 mM Tris/maleate (pH 6.0) and stored at −20°C for several days. It was thawed gradually on ice before use. The ATPase enzyme of cardiac microsomes was partially purified by treating microsomes with deoxycholate, according to the method of MacLennan (1970). \( \text{cAMP}-\text{dependent protein kinase} \) was partially purified from pooled supernatants of cardiac microsomal preparation, that were stored at −20°C until use, through DEAE-cellulose chromatography step by the method of Miyamoto et al. (1969), modified slightly (Kirchberger et al., 1974). The appropriate fractions containing the enzyme activity were dialyzed against 5 mM histidine/HCl buffer (pH 6.8) and stored at −20°C for several months. Pyruvate kinase (EC 2.7.1.40, ATP:pyruvate 2-O-phosphotransferase) was purchased from Oriental Yeast Co., Osaka. Protein transferase) was purchased from Oriental Yeast Co., Osaka. Protein

**Microsomal Phosphorylation Catalyzed by cAMP-dependent Protein Kinase**

Microsomal protein was phosphorylated at 25°C for 2 to 5 min in one of the following procedures.

**Procedure I**—Cardiac microsomes (0.5 to 2 mg of protein/ml) were incubated with 0.25 to 1 mg of cAMP-dependent protein kinase in 1 mM ATP, 1 mM cAMP, 80 mM KCl, and 40 mM histidine/HCl buffer (pH 6.8) in the presence of 2 to 20 mM MgCl₂ in a total volume of 1 to 3 ml (designated as "Medium II"). After 2 to 5 min of incubation at 25°C, 9 volumes of ice cold 10 mM imidazole/HCl buffer (pH 7.0) was added, and the mixture was applied to a column (1.3 x 2 cm) of Dowex 1-X8 (100 to 200 mesh) pre-equilibrated with the same buffer at 0 to 4°C. Microsomes incubated in the presence of EDTA and MgCl₂, which are henceforth designated as control (unphosphorylated) and phosphorylated, respectively, were eluted from the column at a flow rate of about 1 ml/min at 0 to 4°C, and were subjected to assay for the \( \text{Ca}^{2+}\)-dependent "extra" ATP hydrolysis and \( \text{P}^{32} \) incorporation into microsomal ATPase enzyme, as described below. In some experiments, microsomes preincubated without EDTA served as control.

**Procedure II**—Cardiac microsomes (2 mg/ml) were incubated with 1 mg/ml of cAMP-dependent protein kinase in 1 mM ATP, 1 mM cAMP, 80 mM KCl, and 20 mM histidine/HCl buffer (pH 6.8) in the presence of 2 mM MgCl₂, in a total volume of 1 to 3 ml (designated as "Medium II"). After 2 to 5 min of incubation at 25°C, 9 volumes of ice cold 10 mM imidazole/HCl buffer (pH 7.0) was added, and the mixture was applied to a column (1.3 x 2 cm) of Dowex 1-X8 (100 to 200 mesh) pre-equilibrated with the same buffer at 0 to 4°C. Microsomes incubated in the presence of EDTA and MgCl₂, which are henceforth designated as control (unphosphorylated) and phosphorylated, respectively, were eluted from the column at a flow rate of about 1 ml/min at 0 to 4°C, and were subjected to assay for the \( \text{Ca}^{2+}\)-dependent "extra" ATP hydrolysis and \( \text{P}^{32} \) incorporation into microsomal ATPase enzyme, as described below. In some experiments, microsomes preincubated without EDTA served as control.

**Procedure III**—Assay conditions and anion exchange resin treatment were identical with Procedure II, except that \( \gamma\text{-}\text{ATP} \) or \( \gamma\text{-}\text{ATP} \) (1 to 10 \( \mu\text{Ci/mol} \)) for both instead of unlabelled ATP was used (designated as "Medium III"). Aliquots (0.2 ml) were taken immediately before and after the elution, and at timed intervals after the elution, and added to 2 ml of ice cold trichloroacetic acid in order to determine the recovery of radioactivity in the supernatant and whether it was bound to microsomal protein.

**Determination of Phosphorylated Intermediate of \( \text{Ca}^{2+}\)-dependent ATPase**

**Procedure A**—Cardiac microsomes (0.1 mg/ml) were incubated in 10 mM \( \gamma\text{-}\text{ATP} \) (1 to 10 \( \mu\text{Ci/mol} \)), 1 mM MgCl₂, 100 mM KCl, 40 mM histidine/HCl buffer (pH 6.8), 10 mM CaCl₂ (calcium/EGTA buffer), and 5 mM NaN₃ in a total volume of 1 ml (designated as "Medium A") at 0 to 25°C for 0.05 to 30 s. To follow reactions lasting for less than 5 s, a homemade mixing apparatus, which was previously described by Kanazawa et al. (1970), was used. When tested by alkaline hydrolysis of 2,4-dinitrophenyl phosphate or by pyruvate kinase reaction, this apparatus was capable of following the reaction at intervals of 0.05 s. The reaction was started by the addition of radioactive ATP and terminated by the addition of 0.7 to 2 ml of 10% trichloroacetic acid containing 2 mM ATP and 0.5 mM KH₂PO₄, followed by the addition of 1.26 mg of bovine serum albumin/tube as carrier protein. The pellet after centrifugation (1000 X g, 5 min) was washed four more times with 4% perchloric acid containing 30 mM KH₂PO₄, and 10 mM PP, through dilution and centrifugation. Because this phosphoprotein was labile to alkali, we avoided use of alkali during the washing procedure. The washed final pellets were resuspended in 0.2 ml of 0.5 N NaOH, followed by the addition of 1 ml of H₂O, and aliquots were assayed for radioactivity by liquid scintillation spectrometry.

**Procedure B**—Cardiac microsomes were phosphorylated by cAMP-dependent protein kinase and unlabeled ATP and were passed through the anion exchange resin according to Procedure II, in order to remove unreacted ATP and products ADP and P₀, which would otherwise interfere with kinetic properties of ATPase (Tada et al., 1978b). An aliquot (0.5 ml) of resultant eluate was added to 0.2 ml of a solution that gave final concentrations of 1 to 200 \( \mu\text{mol} \) of ATP (10 to 100 \( \mu\text{Ci/mol} \)), 1 mM MgCl₂, 100 mM KCl, 5 mM NaN₃, 10⁻⁴ M ionized CaCl₂ (calcium/EGTA buffer), and 10 mM imidazole/EGTA buffer (pH 7.0) (designated as "Medium B"). The reaction was carried out as described under "Procedure A."

**Assay for ATPase Activity**

Microsomes were incubated under conditions similar to those for the determination of phosphorylated intermediate and reactions were carried out as described under "Procedures A and B."

**Decomposition of Phosphorylated Intermediate of ATPase**

Phosphoprotein intermediate of \( \text{Ca}^{2+}\)-dependent ATPase was formed under conditions described above (Medium A-B) at 0 to 30°C for 2 to 10 s. Further formation of the phosphoprotein intermediate was subsequently terminated by chelation of Ca²⁺ with 9 mM EGTA, and the rate of decay in the amount of the intermediate was determined by the subsequent addition at timed intervals of 0.7 to 1 ml of trichloroacetic acid containing ATP and KH₂PO₄ as described above ("Procedure A"). In the control experiment, H₂O instead of EGTA was added. The time course of decay of the amount of phosphorylated intermediate (EP) was plotted as (EP)/\( \text{EPo} \) or log

One unit (U.) of the enzyme activity is defined as that amount of enzyme that converted 1.0 \( \mu\text{mol} \) of phosphoenolpyruvate to pyruvate per min at pH 7.6 and 30°C.
(\{EY\}'/\{EY\}'_i), where \(\{EY\}'\) and \(\{EY\}'_i\) are the concentrations of \(EY\) at 0 and \(t\) s after the addition of EGTA. Because the decay rate was very rapid, the reaction was followed by the above-mentioned rapid-mixing apparatus (Kanazawa et al., 1970) which was equipped with three reaction channels, allowing the sequential additions of ATP, EGTA, and trichloroacetic acid.

**Stability of Phosphorylated Intermediate of ATPase and Phospholamban Formed by Protein Kinase**

Microsomal phosphorylation in the presence of ionized Ca\(^{2+}\) (Medium A) and cAMP and protein kinase (Medium I) was performed according to “Procedures A and I,” described above. After centrifugation, the pellets were treated with acid, alkali, and hydroxylamine at different temperatures, and the amounts of the remaining phosphoproteins were determined either by washing (“Procedures A and I,” respectively) or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tada et al., 1975a).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**

Gel electrophoresis of microsomal protein was performed according to the procedures of Weber and Osborn (1965), slightly modified (Tada et al., 1975a), on 0.1% sodium dodecyl sulfate, 8 or 10% polyacrylamide gels.

**RESULTS**

**Formation of Two Classes of Phosphoproteins in Cardiac Microsomes—Cardiac microsomes formed two classes of chemically different phosphoproteins. Table I summarizes the typical stability characteristics of these phosphoproteins. The phosphoprotein formed by the reaction of microsomes with \([\gamma-\text{32P}]\text{ATP}\), cAMP, and cAMP-dependent protein kinase was stable in hydroxylamine and in hot acid in accord with previous reports (Kirchberger et al., 1974; Tada et al., 1975a), whereas the other was hydroxylamine-labile acyl phosphoryl protein that was formed when microsomes were incubated with \([\gamma-\text{32P}]\text{ATP}\) and a trace amount of Ca\(^{2+}\). The latter phosphoprotein was shown to represent the phosphorylated intermediate of Ca\(^{2+}\)-dependent ATPase of cardiac sarcoplasmic reticulum (Pang and Briggs, 1973; Shigekawa et al., 1976), which possessed characteristics almost similar to the ATPase enzyme of skeletal muscle sarcoplasmic reticulum (Tada et al., 1978b). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the hydroxylamine-labile acyl phosphoryl protein had a molecular weight of 95,000 to 100,000.

**Table I** Comparison of stability of two kinds of phosphoproteins formed in cardiac microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of phosphorylated intermediate of ATPase (\text{nmol P/mg protein})</th>
<th>Amount of phosphorylated phospholamban (\text{nmol P/mg protein})</th>
</tr>
</thead>
<tbody>
<tr>
<td>None; 0°C (=control)</td>
<td>0.74</td>
<td>100</td>
</tr>
<tr>
<td>None; 0°C</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>0.5 nm NaOH; 25°C</td>
<td>0.62</td>
<td>92.5</td>
</tr>
<tr>
<td>0.5 nm NaOH; 90°C</td>
<td>0.31</td>
<td>46.3</td>
</tr>
<tr>
<td>Control (0.8 nm NaCl); 30°C</td>
<td>0.61</td>
<td>100</td>
</tr>
<tr>
<td>0.8 nm hydroxylamine; 30°C</td>
<td>&lt;0.01</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

whereas the hydroxylamine-stable phosphoprotein exhibited a molecular weight of about 22,000. Although the two kinds of phosphoproteins were formed under two different conditions which were favorable for phosphorylation of each, simultaneous phosphorylation of both proteins could be demonstrated when microsomes were incubated in the presence of millimolar concentrations of ATP and Mg\(_2\)C\(_6\), micromolar concentrations of cAMP and Ca\(^{2+}\), and cAMP-dependent protein kinase at neutral pH and 25°C for a few minutes, provided that use of alkali was avoided during the washing of phosphorylated proteins.

**Effect of Aging on the Formation of Two Phosphoproteins—**When cardiac microsomes were kept on ice after preparation, the amount of the 22,000-dalton phosphoprotein formed by incubation with protein kinase remained almost unchanged (Fig. 1), particularly when the assay was performed in the presence of 25 mm NaF. Incubation in the absence of NaF resulted in the formation of a slightly lower amount of the phosphoprotein when microsomes were relatively fresh. However, the phosphoprotein formation in the absence of NaF increased with aging of the microsomes, and in 48 h of storage, it became almost equal to that in the presence of NaF. These intriguing phenomena, which warrant further investigation, would probably be caused by phosphoprotein phosphatase activity present in cardiac microsomes (Tada et al., 1975b). Fig. 1 also indicates that the amount of phosphoprotein intermediate of ATPase decreased precipitously upon aging, becoming less than half with 20 h after preparation. The decrease could be prevented to some extent by freezing the sample, but not by freeze-drying, in the presence of sucrose, provided that the sample was thawed gradually on...
ice. We attempted to prevent the rapid deterioration of the intermediate by adding agents like dithiothreitol and protease inhibitors to the preparation without much success. Therefore, in the subsequent experiments to determine the amount of intermediate, all assays were performed within several hours after preparation was completed, unless otherwise stated.

Effects of cAMP-dependent Protein Kinase on Ca\(^{2+}\)-dependent ATPase of Cardiac Sarcoplasmic Reticulum—In examining the mechanism of the stimulatory effect of protein kinase and cAMP on the enzyme kinetics of ATPase, special care was taken not to bring unreacted ATP and products ADP and P\(_i\) formed during pretreatment of microsomes with protein kinase into the ATPase assay media, since these would otherwise interfere with enzymatic properties of the ATPase (Tada et al., 1978b) and significantly decrease the specific radioactivity of \([\gamma-\text{32P}]\text{ATP}\), thus making the results equivocal. Virtually all of the nucleotides and P\(_i\) were removed by passing the reaction mixture through Dowex 1-X8 resin, when monitored by the parallel experiments using \([\gamma-\text{32P}]\text{ATP}\) and \([2,8-\text{3H}]\text{ATP}\) ("Procedure III," see "Experimental Procedures"). When kept on ice, the amount of \(32\text{P}\) bound to the 22,000-dalton protein (0.6 to 0.8 nmol of phosphate/mg microsomal protein), the incubation under identical conditions ("Procedure II and III") resulted in significant phosphorylation of the 22,000-dalton protein (0.5 to 0.8 nmol of phosphate/mg microsomal protein), the incubation under identical conditions in the presence of 2 mM EDTA instead of MgCl\(_2\) gave virtually no phosphorylation.

To examine whether the preincubation of microsomes with EDTA can serve as a suitable control for preincubation with MgCl\(_2\) in the determination of protein kinase effects on the activity of ATPase enzyme, cardiac microsomes were incubated without or with 2 mM EDTA or with 2 mM MgCl\(_2\) at

![Table II](http://www.jbc.org/)

**Table II**

Effects of pretreatment with EDTA and MgCl\(_2\) on the Ca\(^{2+}\)-dependent ATPase activity of cardiac microsomes

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Preincubation time</th>
<th>pH</th>
<th>nmol pyruvate/mg-min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5 min</td>
<td>6.8</td>
<td>12.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>6.8</td>
<td>12.7</td>
</tr>
<tr>
<td>MgCl(_2), 2 mM</td>
<td>5 min</td>
<td>6.8</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>6.8</td>
<td>12.8</td>
</tr>
<tr>
<td>EDTA, 2 mM</td>
<td>5 min</td>
<td>6.8</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>6.8</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>8.0</td>
<td>19.1</td>
</tr>
<tr>
<td></td>
<td>20 min</td>
<td>8.0</td>
<td>25.5</td>
</tr>
</tbody>
</table>

* Original microsomes with no preincubation.

* Mean ± S.E. (\(n = 6\)).

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Effect of treatment with cAMP and protein kinase on Ca\(^{2+}\)-dependent ATP hydrolysis and formation of phosphorylated intermediate EP of ATPase. Cardiac microsomes (2.0 mg/ml) were preincubated in Medium II with 1.0 mg/ml of cAMP-dependent protein kinase in the presence of 2 mM EDTA (open symbols) and 2 mM MgCl\(_2\) (closed symbols) at 25°C for 5 min in a total volume of 1 ml. After application of the diluted reaction mixture to a column of Dowex 1-X8 at 0 to 4°C, as described under "Procedure II," the eluted microsomes were incubated in Medium B in the presence of 10 μM \([\gamma-\text{32P}]\text{ATP}\) and 10 μM ionized Ca\(^{2+}\) at 15°C. Microsomal protein concentration in the final reaction mixture was 0.14 mg/ml. The amount of P\(_i\) liberated (△, □) and EP formed (○, ●) by cardiac microsomes was determined as described under "Experimental Procedures." Cardiac microsomes, preincubated with \([\gamma-\text{32P}]\text{ATP}\) and protein kinase in Medium III, i.e., under conditions identical with Medium II, in the presence of 2 mM EDTA and 2 mM MgCl\(_2\), exhibited phosphorous phosphorylation of 0.02 and 0.61 μmol of phosphate/g of microsomal protein, respectively, as determined by washing with dilution and centrifugation (see "Experimental Procedures").

At pH 6.8 and 25°C, according to "Procedure II," except that cAMP and cAMP-dependent protein kinase were omitted (Table II). The incubation with EDTA at pH 6.8 up to 20 min produced virtually no alteration in the ATPase activity, compared with that without EDTA or with MgCl\(_2\), whereas the incubation with EDTA at higher pH (8.0) resulted in a significant increase in the ATPase activity, in accord with reports by Duggan and Martonosi (1970) and MacLennan (1974). These findings indicate that a brief (2- to 5-min) incubation of cardiac microsomes with EDTA at pH 6.8, i.e., the conditions under which the microsomal membranes were reacted with protein kinase ("Procedure II"), does not significantly affect the ATPase enzyme, and thus could suitably serve as a control for preincubation with MgCl\(_2\).

Fig. 2 typically represents time courses of P\(_i\) liberation and formation of phosphorylated intermediate (EP), catalyzed by control and phosphorylated cardiac microsomes at 15°C in the presence of 10 μM \([\gamma-\text{32P}]\text{ATP}\) and 10 μM ionized Ca\(^{2+}\). It was evident that in the presence of saturating concentration of ionized Ca\(^{2+}\) (10 μM), the steady state levels of EP were not
Fig. 3. Effect of treatment with cAMP and protein kinase on the ATP dependence profile of the Ca²⁺-dependent ATPase activity of cardiac microsomes. Cardiac microsomes (2 mg/ml) were preincubated in Medium II with 1 mg/ml of cAMP-dependent protein kinase in the presence of 2 mM EDTA (○) or 2 mM MgCl₂ (●) at 25°C for 5 min in a total volume of 3 ml and were passed through a column of Dowex 1-X8 after dilution with buffer solution, as described under "Procedure II." The resultant eluates were incubated in Medium B in the presence of 10 μM ionized Ca²⁺, 20 μL of pyruvate kinase, 2.5 mM phosphoenolpyruvate, and 1 to 200 μM unlabeled ATP. Microsomal protein concentration in the final reaction mixture was 0.12 mg/ml. The amount of pyruvate liberated was determined by the procedures of Reynaud et al. (1961). The inset represents Lineweaver-Burk plots of the same experiment. Cardiac microsomes, preincubated with protein kinase and [γ-32P]ATP under identical conditions (Medium III) in the presence of 2 mM EDTA and 2 mM MgCl₂, exhibited phosphorylation of 0.015 and 0.76 nmol/mg of phosphoester phosphorylation of protein, respectively, significantly altered by the phosphorylation of phospholamban, whereas the rate of P_i liberation was markedly enhanced by the phosphorylation. Similar results were obtained at higher ATP concentrations up to 1 mM. In the presence of 0.5 mM EGTA, however, neither the rate of P_i liberation nor the steady state levels of EP was significantly affected by phosphorylation of phospholamban, as was reported previously (Tada et al., 1974).

Effect of Phospholamban Phosphorylation on ATP Dependence Profile of Ca²⁺-dependent ATPase Activity—Since the Ca²⁺-dependent ATPase activity of cardiac microsomes, like that of its skeletal muscle counterpart (Tada et al., 1976b), was shown to exhibit a complex MgATP dependence (Shige-kawa et al., 1976), we examined the effect of phospholamban phosphorylation on the ATP concentration dependence of cardiac microsomal ATPase. In these experiments, the ATP concentrations were maintained by inclusion of pyruvate kinase and phosphoenolpyruvate in the media as the ATP regenerating system, and the rates of ATP hydrolysis were determined by measuring the liberation of pyruvate. Fig. 3 shows a typical experiment in which the ATP concentration was varied between 1 and 200 μM in the presence of 10 μM ionized Ca²⁺ at 15°C. The Ca²⁺-dependent ATPase activity increased with increasing concentration of ATP, and the stimulatory effect of phospholamban phosphorylation on the ATPase was more evident at higher ATP concentrations. Essentially similar results were obtained when microsomes preincubated without EDTA were used as control. A Lineweaver-Burk plot of the same experiment (inset, Fig. 3) indicated different slopes at the low and high ATP concentration ranges. It was evident that phosphorylation of phospholamban caused a marked downward shift of the double reciprocal plots. The values of K_M and the maximal velocity, V_max, of the ATPase of control microsomes calculated from the linear plots are 2.5 μM and 11.5 nmol/mg/min, respectively, at the low ATP concentration range (1 to 5 μM), and 17.2 μM and 18.1 nmol/mg/min, respectively, at the high ATP concentration range (10 to 200 μM). The corresponding values for phosphorylated microsomes were 2.4 and 1.9 μM and 37.2 and 37.7 nmol/mg/min, respectively, at the low and high ATP concentration ranges. Table III compares the V_max values of control and phosphorylated microsomes in another set of experiments (see below).

Effect of Phospholamban Phosphorylation on Ca²⁺ Dependence Profile of EP Formation and P_i Liberation—Since the observed enhancement of the cardiac microsomal Ca²⁺-dependent ATPase activity could accompany alterations in the intermediary steps of the ATPase reaction, in view of the well-documented reaction scheme of its skeletal muscle counterpart (Kanazawa et al., 1971; Tada et al., 1978b), we examined effects of phospholamban phosphorylation on the Ca²⁺ dependence profile of the steady state levels of EP, the rate of P_i liberation (v), and the ratio v/[EP], i.e., the ratio of the rate of P_i liberation to the concentration of EP (Fig. 4). Within a range of ionized Ca²⁺ between 0.1 to 10 μM, the rate of P_i liberation was markedly higher in phosphorylated microsomes, whereas the steady state levels of EP were significantly lower in phosphorylated microsomes. Thus, the ratio v/[EP], which was independent of ionized Ca²⁺, increased more...
Cyclic AMP Regulation of Ca\(^{2+}\)-ATPase

FIG. 4. Effect of treatment with cAMP and protein kinase on the Ca\(^{2+}\) dependence profiles of the rate of Pi liberation and the formation of intermediate of ATPase (EP). Cardiac microsomes (2 mg/ml) were incubated in Medium II with 1 mg/ml of cAMP-dependent protein kinase in the presence of 2 mM EDTA (A, open symbols) and 2 mM MgCl\(_2\) (B, closed symbols) at 25°C for 3 min in a total volume of 3 ml and were passed through a column of Dowex 1-X8 after dilution with buffer solution, as described under “Procedure II.” The resultant eluates were incubated in Medium B in the presence of 100 \(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP and 0.01 to 20 \(\mu\)M ionized Ca\(^{2+}\) at 15°C for different time intervals (5 to 30 s). Microsomal protein concentration in the final reaction mixture was 0.14 mg/ml. The rate, \(v\), of Pi liberation (A, △) and the amount of EP formed (O, O) were determined as described under “Experimental Procedures.” The ratio \(v/\text{[EP]}\) (×) at each Ca\(^{2+}\) concentration was determined by dividing the value of \(v\) by the concentration of EP (\([\text{EP}]\)) at corresponding Ca\(^{2+}\) concentration.

Cardiac microsomes, preincubated with protein kinase and [\(\gamma\)-\(^{32}\)P]ATP in Medium III in the presence of 2 mM EDTA and 2 mM MgCl\(_2\), exhibited phosphoester phosphorylation of 0.018 and 0.77 pmol of phosphate/g of microsomal protein, respectively. \(pCu\), –log[ionized Ca\(^{2+}\)].

Fig. 5. Effect of treatment with cAMP and protein kinase on the decomposition of the phosphoprotein intermediate (EP) of Ca\(^{2+}\)-dependent ATPase of cardiac sarcoplasmic reticulum. Following pretreatment with protein kinase and with anion exchange resin, as in Fig. 5, control (○) and phosphorylated (●) cardiac microsomes were incubated with 10 \(\mu\)M CaCl\(_2\) in Medium B in the presence of 100 \(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP at temperatures ranging from 0 to 30°C. Ten seconds after the addition of Ca\(^{2+}\), further formation of EP was terminated by chelation of Ca\(^{2+}\) with 2 mM EGTA, and the rate of decay in the amount of EP was determined by the subsequent addition of trichloroacetic acid at timed intervals, as described under “Experimental Procedures.” The logarithm of the first order rate constant, \(k_d\), of EP decomposition was plotted against reciprocal temperature.

Enhancement of Rate of EP Decomposition by cAMP-dependent Protein Kinase-catalyzed Phosphorylation—In view of the remarkable augmentation of the ratio \(v/[\text{EP}]\) by phospholamban phosphorylation, we examined whether phospholamban phosphorylation produces an increase in the rate of decay in the amount of EP when further formation of EP was terminated by the addition of excess EGTA. As seen in Fig. 5, the rate of EP decomposition of phosphorylated microsomes was about 2-fold higher than that of control. When the temperature dependence of the first order rate constant, \(k_d\), of EP decomposition was studied, it was evident that the \(k_d\) value of phosphorylated microsomes was markedly higher than that of control microsomes within a wide range of the temperature between 0 and 30°C (Fig. 6). The Arrhenius plots for \(k_d\) of both control and phosphorylated microsomes indicated different slopes below and above 18°C. The activation energies of the phosphorylated microsomes calculated from these plots were 11.4 and 19.2 kcal/mol at high and low than 2-fold when microsomes were phosphorylated by cAMP-dependent protein kinase. It should be noted that phospholamban phosphorylation produced profound reduction in EP levels at lower Ca\(^{2+}\) concentrations while the effect was less evident at higher Ca\(^{2+}\) concentrations.
temperatures, respectively, and were almost similar to those of control microsomes.

DISCUSSION

The marked stimulation of Ca$^{2+}$-dependent ATPase activity of cardiac sarcoplasmic reticulum by cAMP and cAMP-dependent protein kinase was suggested to be mediated by phosphorylation of the 22,000-dalton microsomal protein (phospholamban), on the basis of observations that the overall rates of calcium uptake and Ca$^{2+}$-dependent ATP hydrolysis were markedly enhanced when cAMP-dependent protein kinase catalyzes phosphorylation of phospholamban (Tada et al., 1974, 1975a; Kirchberger et al., 1974; Kirchberger and Tada, 1976) and these effects were abolished by the treatment of microsomes with protein phosphatase (Kirchberger and Raffo, 1977) and protein kinase inhibitor (Tada et al., 1977b). While these studies may raise the possibility that phospholamban can function as a regulatory factor controlling the active calcium transport by cardiac sarcoplasmic reticulum (Katz et al., 1975; Tada et al., 1978), it was still unclear whether the Ca$^{2+}$-dependent ATPase enzyme of cardiac sarcoplasmic reticulum, which is known as an energy transducer of calcium transport in these membranes (Tada et al., 1978b), could directly be affected by the phosphorylated and unphosphorylated states of phospholamban, and, if so, whether one or more of the elementary steps of the ATPase reaction is influenced by the latter protein. In the present study, in which the phosphorylated and unphosphorylated states of phospholamban were carefully controlled by monitoring the amounts of phosphoester phosphorylation in the parallel reaction mixture (Medium III), it was demonstrated that the enzymatic parameters of the Ca$^{2+}$-dependent ATPase of cardiac sarcoplasmic reticulum undergo profound alterations when cAMP-dependent protein kinase catalyzes phosphorylation of the microsomal protein phospholamban. These alterations include about 2-fold increases, over control, in the values of $V_{max}$, V/$[E_P]$, and $k_2$ (Table III), suggesting that the probable site of action of phospholamban is an intermediary step of the ATPase at which the reaction intermediate is decomposed.

The observed enzymatic alterations are not considered to represent nonspecific changes like those induced by mere exposure of membranes to different ionic conditions during pretreatment with kinase, nor are they due to a change during the resin treatment. Thus, in the control experiments in which membranes were preincubated with or without EDTA and MgCl$_2$ at different pH, it was found that the ATPase activity does not undergo a significant change upon incubation at neutral pH, but it does upon that with EDTA at alkaline pH (Table I). The latter observations are in good agreement with findings on skeletal muscle microsomes by Duggan and Mar-tonosi (1970) and MacLennan (1974) who showed that the ATPase activity is considered to form an essential part of the active calcium transport system in skeletal muscle sarcoplasmic reticulum (MacLennan and Holland, 1975; Tada et al., 1978b), and an essentially similar mechanism was shown to be applicable to cardiac muscle sarcoplasmic reticulum (Pang and Briggs, 1973; Shigekawa et al., 1976). In the former, the translocation of calcium ions from outside to inside the membrane is closely associated with the formation and decomposition of the reaction intermediate $E'$ in the manner summarized in the following equation (Tada et al., 1978b):

$$E + 2Ca^{2+} + ATP + [E_P]_{Ca} \rightarrow E + P_0 + 2Ca^{2+}$$

where $i$ and $o$ indicate the inside and outside of the membrane vesicles, respectively; $E$ represents the ATPase enzyme. At the outer surface of the membrane, 2 mol of Ca$^{2+}$ and 1 mol of ATP bind 1 mol of $E$ to form the Michaelis complex $E_iCa^{2+}ATP$. This is immediately followed by the formation of the phosphorylated intermediate $E_{P}Ca^{2+}$ when calcium is translocated from outside to inside the membrane. Calcium is subsequently released from the enzyme to the interior of the vesicle, with the simultaneous decomposition of the intermediate into $E$ and $P_0$. Among these intermediary steps, Step i is considered to be rate-determining in the presence of saturating concentration of ATP and Ca$^{2+}$ (Tada et al., 1978b) and could be followed by conventional methods (see “Experimental Procedures”), whereas Steps ii and iii are not the rate-determining steps in the presence of saturating concentrations of ATP and Ca$^{2+}$ (Tada et al., 1978b) and are too rapid to be followed by conventional quenching apparatus. In practice, therefore, we mainly aimed in the present study at examining the rate constant of Step iii by determining the ratio of the rate ($v$) of $P_0$ liberation to the concentration of $E_iCa^{2+}ATP$ (referred to as $E_0$ throughout the present report), as well as by directly measuring the rate of decay in the amount of $E_0$, and compared these with the overall rate of ATP hydrolysis.

The present demonstration that $V_{max}$ of ATP hydrolysis is markedly enhanced by protein kinase-catalyzed phosphorylation (Fig. 3 and Table III) is consistent with the previous observations that the overall rates of production of $P_0$ and accumulation of Ca$^{2+}$ are greatly enhanced by protein kinase with the maintenance of the stoichiometric coupling of 2 mol of calcium taken up per mol of $E_0$ hydroyzed (Tada et al., 1974, 1975a; Kirchberger et al., 1974; Kirchberger and Tada, 1976; LaRaia and Morkin, 1974; Schwartz et al., 1976; Wray and Gray, 1977) and thus suggests that the ATPase enzyme itself, but not the efficiency of coupling, is directly affected by protein kinase-catalyzed phosphorylation. Enhancement of any of the steps in Equation 1 can account for the observed stimulation. No significant alteration in the rate of $E$ formation (Steps i plus ii in Equation 1) was indicated when control and phosphorylated microsomes were incubated with radioactive ATP in the presence of ionized Ca$^{2+}$ for 0.05 to 0.1 s, the shortest time intervals our apparatus (see “Experimental Procedures”) can follow. However, in view of an extreme rapidity at which $E'$ is formed (Kana zawa et al., 1971; Froehlich and Taylor, 1975), it still remains to be examined by high

1 M. Tada, M. Yanada, F. Ohmori, and H. Abe, unpublished observations.
performance apparatus whether the rate of EP formation is altered by phospholamban phosphorylation. In contrast, the rate of EP decomposition (Step iii in Equation 1) could be followed either by determining the ratio \( v/[\text{EP}] \) or by directly measuring the specific rate constant \( k_v \) of EP decomposition (Tada et al., 1978b). We found that both the ratio \( v/[\text{EP}] \) and the \( k_v \) value of phosphorylated microsomes are to the same extent, e.g., 2.1- and 1.9-fold, respectively (Table III), higher than those of control microsomes, being in accord with thermodynamic analysis of this system (Tada et al., 1978b). These findings indicate that protein kinase-catalyzed phosphorylation of phospholamban enhances the rate of EP decomposition. Such enhancement is presumably more effective in reducing the steady state levels of EP at lower \( \text{Ca}^{2+} \) concentration ranges (Fig. 4), where the EP formation (Step i or ii in Equation 1, or both), rather than the EP decomposition (Step iii), is the rate-determining step, whereas no effect on those is seen at higher \( \text{Ca}^{2+} \) concentration ranges (Fig. 4), where the \( \text{Ca}^{2+} \) is decomposed, the resultant increase in the rate of turnover of the enzyme reaction would account for about 2-fold increases in both \( V_{\text{max}} \) for \( \text{Ca}^{2+} \)-dependent ATP hydrolysis (Fig. 3 and Table III) and the rate of calcium transport (Tada et al., 1974; Will et al., 1976; Schwartz et al., 1976) when cardiac microsomes were subjected to phosphorylation by cAMP-dependent protein kinase.

Thus, the present report indicates that cAMP-dependent protein kinase can control the rate of \( \text{Ca}^{2+} \)-dependent ATPase by regulation of the rate of EP decomposition and that this process may be associated with phosphorylation of phospholamban, a microsomal substrate for protein kinase. Such a conclusion is in accord with the view that phospholamban serves as a regulator controlling the \( \text{Ca}^{2+} \)-dependent ATPase (Tada et al., 1975a; 1978a). While the probable site of action of phospholamban on the ATPase is shown to be that at which EP is formed, it remains to be determined whether the step(s) at which EP is formed is also enhanced. It also remains to be seen whether the observed increases in the enzymatic parameters are induced by the direct molecular interactions between phospholamban and the ATPase or whether it is caused by an indirect effect such as that involving the lipid-protein interactions within the membrane. Preliminary results are suggestive of the former possibility (Tada et al., 1979).

In view of the role of cAMP and cAMP-dependent protein kinase in the regulation of active calcium transport by cardiac sarcoplasmic reticulum, it is of importance to determine intracellular localization of these within cardiac cells. cAMP was shown by Ong and Steiner (1977) to localize in the area of sarcoplasmic reticulum and sarcosomes. Corbin et al. (1977) suggested that cAMP-binding regulatory subunit of protein kinase is bound to the membrane, and the active catalytic subunit is released by formation of a complex of cAMP and regulatory subunit when intracellular cAMP levels increase after hormonal intervention. Examination of possible compartmentalization of cAMP, protein kinase, and sarcoplasmic reticulum remains a challenge for future investigation.

Acknowledgments—We are greatly indebted to Drs. Yuji Tonomura and Taibo Yamamoto for their valuable comments and suggestions during the course of this study and during preparation of the manuscript.
Mechanism of the stimulation of Ca2+-dependent ATPase of cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. Role of the 22,000-dalton protein.

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