Transient State Kinetic Studies of Ca\(^{2+}\)-dependent ATPase and Calcium Transport by Cardiac Sarcoplasmic Reticulum

**EFFECT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE-CATALYZED PHOSPHORYLATION OF PHOSPHOLAMBN***

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Phosphorylation of the 22,000-dalton protein phospholamban of cardiac microsomes (sarcoplasmic reticulum) by cAMP-dependent protein kinase was shown to alter profoundly the intermediary steps of microsomal Ca\(^{2+}\)-dependent ATPase. To define the control of ATPase by its putative regulator phospholamban, we examined transient kinetics of the formation of the phosphorylated intermediate EP of ATPase and calcium binding by the membrane through a rapid quenching device, after microsomes were incubated with and without cAMP-dependent protein kinase. When microsomes were suspended with ethylene glycol bis(β-aminoethyl ether)N\(_2\)N\(_2\)-tetraacetic acid (EGTA) prior to the assay (Ca\(^{2+}\)-free microsomes), the initial rates of EP formation and calcium binding were markedly enhanced in phosphorylated microsomes, whereas those incubated with calcium/EGTA buffer (Ca\(^{2+}\)-bound microsomes) exhibited less pronounced enhancement. In Ca\(^{2+}\)-free microsomes, these effects were evident within a wide range of ionized Ca\(^{2+}\) between 0.1 and 20 \(\mu\)M. Under these conditions, the initial rates and maximal amounts of EP formation were enhanced significantly by microsomal phosphorylation above 10 \(\mu\)M ATP, while those were reduced at lower ATP (1 to 5 \(\mu\)M). Latter effects were attributed to the predominance of a protein kinase-induced increase in EP decomposition at lower ATP ranges, where the EP formation is rate-determining. These results indicate that cAMP-dependent phosphorylation of phospholamban produces marked increases in the rate of a step associated with calcium binding to the ATPase enzyme and the rate at which EP is subsequently formed, resulting in an increased rate at which calcium is translocated across the microsomal membrane. Together with our previous report (Tada, M., Ohmori, F., Yamada, M., and Abe, H. (1979) J. Biol. Chem. 264, 319–324) that phospholamban phosphorylation induces a marked increase in the rate of EP decomposition, the present observations suggest that phospholamban, through modulation of elementary steps of ATPase, could function as a regulator controlling the active calcium transport by cardiac sarcoplasmic reticulum.

Our previous report (3) indicated that cAMP-dependent protein kinase\(^{1}\) (EC 2.7.1.37, ATP:protein phosphotransferase)-catalyzed phosphorylation of a 22,000-dalton protein phospholamban of cardiac sarcoplasmic reticulum results in increased Ca\(^{2+}\)-dependent ATPase activity (EC 3.6.1.3, ATP phosphohydrolase) of cardiac sarcoplasmic reticulum, through an enhancement of the rate of decomposition of the phosphorylated intermediate EP of the ATPase. These observations suggest that the phosphorylated and unphosphorylated states of phospholamban (4) can determine the extent at which the elementary step(s) of Ca\(^{2+}\)-dependent ATPase is stimulated, and are thus in support for the view that phospholamban may function as a regulatory factor controlling the active calcium transport by cardiac sarcoplasmic reticulum (5–7). This proposal was consistent with a number of findings that the rates of calcium transport and Ca\(^{2+}\)-dependent ATP hydrolysis can be augmented when cAMP-dependent protein kinase catalyzes phosphorylation of a microsomal protein of 20,000 to 22,000 daltons (4, 8–16). Resultant increases in the rate and amount of calcium accumulation into sarcoplasmic reticulum may account for the relaxing and inotropic responses of the myocardium when exposed to agents that increase intracellular Ca\(^{2+}\) levels (5, 6, 17, 18).

Active calcium transport by sarcoplasmic reticulum from skeletal and cardiac muscles is considered to be mediated by an ATPase enzyme of about 100,000 daltons, which serves as an energy transducer and a translocator of calcium ions, undergoing rapid and complex series of elementary steps in enzyme states during the translocation of calcium across the membrane (19). Our previous study demonstrated that cAMP-dependent phosphorylation of phospholamban increases the rate at which the intermediate (EP) of the ATPase is decomposed (3). However, in view of the extreme rapidity at which EP is formed (20–23), it is absolutely necessary to examine by employing a high performance rapid mixing device whether the rate of EP formation, which takes place within tens of milliseconds (19), is enhanced by cAMP-dependent phosphorylation of phospholamban. It is also necessary to determine whether such alterations in enzymatic events could accom-

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\(^{1}\) The abbreviations used are: protein kinase, adenosine 3':5'-monophosphate-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)N\(_2\)N\(_2\)-tetraacetic acid; EP, the phosphorylated intermediate of Ca\(^{2+}\)-dependent ATPase of cardiac sarcoplasmic reticulum; pCa, – log [ionized Ca\(^{2+}\)].
pany a change in the rate of translocation of calcium ions.

We therefore investigated in the present paper the transient state kinetic properties of Ca\(^{2+}\)-dependent ATPase and calcium transport by cardiac sarcoplasmic reticulum microsomes. Phospholamban is at unphosphorylated and phosphorylated states, through a chemical quench flow apparatus, originally designed by Froehlich et al. (24) to meet measurements of extremely rapid reactions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cardiac microsomes, which consist largely of fragmented sarcoplasmic reticulum, were prepared from dog heart ventricle by the procedure of Harigaya and Schwartz (25) with slight modifications (3, 11). Microsomes were stored on ice and used within several hours after preparation. CAMP-dependent protein kinase was prepared as described previously (3). Protein concentrations were measured by the method of Lowry et al. (26). \(^{14}C\)ATP (20 to 35 mCi/\(\mu\)mol) and \(^{14}C\)CaCl\(_2\) (2 to 3 mCi/\(\mu\)mol) were purchased from New England Nuclear or the former was synthesized by the method of Martin and Doty (3).

**Calcium-binding Studies**—

Cardiac microsomes obtained from Japan Radioisotope Association, Tokyo, were filtered through Millipore filters to remove fine particles and stored on ice. The resultant eluate gave concentrations of 3 to 5 mg of protein/ml. After 2 to 5 min of incubation at 25°C, 4 volumes of ice-cold 20 mM Tris/maleate (pH 6.8) was added and the mixture was applied to a column of Dowex 1-X8 (1.5 x 2 cm) pre-equilibrated with the same buffer at 0-4°C. Microsomes preincubated without or with protein kinase, which are henceforth designated as control (unphosphorylated) and phosphorylated microsomes, respectively, were eluted from the column at a flow rate of 1 ml/min at 0-4°C, and subjected to a formaldehyde (10 to 20 mM) lysis, as described below. In some experiments, microsomes preincubated with 2 mM EDTA and protein kinase served as a control. When incubated with cardiac microsomes and \(^{32}P\)ATP by Procedure 1, the incubation with protein kinase resulted in significant phosphorylation of phospholamban (0.6 to 0.8 \(\mu\)mol of phosphate incorporated/g of microsomal protein), whereas the incubation under identical conditions without protein kinase or with EDTA and protein kinase gave virtually no phosphorylation (<0.2 \(\mu\)mol of phosphate/g).

**RESULTS**

**Effect of cAMP-dependent Protein Kinase on Transient Kinetics of Ca\(^{2+}\)-ATPase**—Cardiac microsomes were phosphorylated by cAMP-dependent protein kinase and unlabelled ATP and were passed through the anion exchange resin according to Procedure II (3), as described above, in order to remove unreacted ATP and products ADP and P\(_i\), which were then combined with kinase and ATPase activity in a resuspending buffer. The resultant eluate (10 to 20 ml) was added 8 ml of a solution that gave concentrations of 3 mM MgCl\(_2\), 100 mM KCl, 5 mM Na\(_2\)ATP, and 20 mM Tris/maleate (pH 6.8). The final concentrations of these in the ATPase assay medium were maintained by the inclusion of a vehicle solution in the starting reaction mixture, which contained 20% trichloroacetic acid with ATP and 5 mM KH\(_2\)PO\(_4\). The standard vehicle solution for the enzyme and substrate solutions contained 100 mM KCl, 3 mM MgCl\(_2\), 5 mM Na\(_2\)ATP, and 20 mM Tris/maleate (pH 6.8), unless otherwise stated. The temperature was maintained at 25 ± 0.5°C by a constant temperature circulator. In some experiments, the enzyme solutions contained cardiac microsomes and calcium/EGTA buffers which gave various concentrations of ionized Ca\(^{2+}\), and the substrate solution contained [y-\(^32\)P]ATP and calcium/EGTA buffer, whose concentration was identical with that in the enzyme solution. For the experiments to determine phosphorylated enzyme (EP), a constant volume (1.8 to 2.0 ml) of the assay solution was added to a solution containing 1.26 mg of bovine serum albumin, and the amount of \(^32\)P incorporated was determined by washing with perchloric acid, as described previously (3). The amount of \(^32\)P liberated was determined by thin-layer chromatography of the reaction products, as described previously (3).

Henceforth, cardiac microsomes with EGTA in the enzyme solution will be designated as Ca\(^{2+}\)-free microsomes (enzyme), and those with calcium/EGTA buffer will be designated as Ca\(^{2+}\)-bound microsomes (enzyme).

**Ca\(^{2+}\)-ATPase Assay with Replacing Unlabeled ATP**—In some experiments, cardiac microsomes were incubated with \(^{32}P\)ATP, and the enzyme and substrate solutions were mixed together at 20°C in the first mixer, and after various times (5 to 200 ms) were quenched with a solution containing 6 mM EGTA in the EGTA solution. Immediately after quenching, 2 ml of the assay solution was passed through a Millipore filter, and the precipitate with retained \(^45\)Ca was washed in 1 ml EGTA in 50 mM Tris/maleate (pH 6.8) and 100 mM KCl at 0-4°C. After drying the Millipore filter was digitized with a liquid scintillation counter and counted for radioactivity.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Cardiac microsomes of phosphorylated or unphosphorylated microsomes were then performed according to the procedures of Weber and Osborn (31), with slight modifications (4), on 0.1% sodium dodecyl sulfate and 8% polyacrylamide gels.

**RESULTS**

Effect of cAMP-dependent Protein Kinase on Transient State Time Courses of Ca\(^{2+}\)-dependent EP Formation and Calcium Binding—A brief incubation of cardiac microsomes with cAMP-dependent protein kinase resulted in marked enhance-
Protein Kinase Effects on Transient Kinetics of Ca\textsuperscript{2+}-ATPase

Effect of CAMP-dependent Phosphorylation on Ca\textsuperscript{2+} Dependence Profile of EP Formation—When the initial rate of EP formation and the maximal amount of EP formed were examined as function of pCa in Ca\textsuperscript{2+}-free enzyme (Fig. 3), the employing Ca\textsuperscript{2+}-free microsomes, whose time courses followed the first order kinetics. Also, in analyzing the initial rates of EP formation and calcium binding, they were assessed by determining directly (micromoles of phosphate and calcium per g of protein in 10 ms) by taking the initial few points during the rise of the time courses, rather than taking the $t_{\frac{1}{2}}$ values, because the latter did not represent the true value due to a significant increase in the levels of EP formed and calcium bound in phosphorylated microsomes (Figs. 1 and 2). It should also be added that a brief incubation of microsomes with different concentrations of EGTA at pH 6.8, conditions under which microsomes were kept in the enzyme syringe in studies with Ca\textsuperscript{2+}-free microsomes, did not appreciably alter the ATPase and calcium-binding activities of the microsomes for considerable period of time (up to 2 h).

**Figure 1.** Effect of treatment with CAMP-dependent protein kinase on the initial rate of Ca\textsuperscript{2+}-dependent formation of phosphorylated intermediate EP of ATPase of Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound cardiac microsomes. Cardiac microsomes (7.8 mg/ml) were preincubated under standard conditions with (closed symbols) and without (open symbols) 3.9 mg/ml of CAMP-dependent protein kinase at 25°C for 2 min in a total volume of 3.0 ml. After application of the diluted reaction mixture to a column of Dowex 1-X8 at 0-4°C, the eluted microsomes were added to the standard vehicle solution containing EGTA (C, □) or calcium/EGTA buffer (C, △), respectively, through the rapid chemical quench flow apparatus, as described under “Experimental Procedures.” Microsomal protein concentration in the final ATPase assay medium was 0.41 mg/ml. The amount of EP formed was determined as described previously. Cardiac microsomes, preincubated with [\textgamma\textsuperscript{32}P]ATP and protein kinase under conditions identical with the preincubation prior to the ATPase assay, exhibited phosphate phosphorylation of 0.63 μmol of phosphate/g of microsomal protein, whereas those preincubated without protein kinase gave 0.02 μmol of phosphate/g of microsomal protein.

(43 ms). Fig. 2 shows time courses of calcium binding in the same microsomal preparation under identical conditions. In agreement with the data on EP formation (Fig. 1), CAMP-dependent phosphorylation produced significant increases in the initial rates of calcium binding in both Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound microsomes. However, in the latter microsomes the calcium initially bound by the enzyme (at time zero) before ATP had been added contributed to increase the total amount of calcium binding in both control and phosphorylated microsomes. The $t_{\frac{1}{2}}$ values in both cases exhibited marked shortening by CAMP-dependent phosphorylation. They were 21 ms for phosphorylated microsomes and 38 ms for control in Ca\textsuperscript{2+}-free microsomes; corresponding values in Ca\textsuperscript{2+}-bound microsomes were 12 and 19 ms.

It is of significance to point out that the apparent stoichiometry between the amount of EP formed and calcium bound during the initial period was maintained at about 1:2, in agreement with the kinetic analysis of this system (19), as long as the both determinations were performed simultaneously in the same preparation. Thus, when the data in Figs. 1 and 2 are compared, the initial rates of EP formation and calcium binding in both Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}-bound microsomes showed approximately 1:2 stoichiometry, and it was well maintained after augmentation by CAMP-dependent phosphorylation (Table I).

In view of the relative complexity of transient time courses in Ca\textsuperscript{2+}-bound microsomes, in which EP was formed rapidly and was decomposed precipitously and calcium binding was obscured by the calcium initially bound by microsomes (Figs. 1 and 2), the following studies to analyze the initial rates of EP formation and calcium binding were largely performed by employing Ca\textsuperscript{2+}-free microsomes, whose time courses followed the first order kinetics. Also, in analyzing the initial rates of EP formation and calcium binding, they were assessed by determining directly (micromoles of phosphate and calcium per g of protein in 10 ms) by taking the initial few points during the rise of the time courses, rather than taking the $t_{\frac{1}{2}}$ values, because the latter did not represent the true value due to a significant increase in the levels of EP formed and calcium bound in phosphorylated microsomes (Figs. 1 and 2). It should also be added that a brief incubation of microsomes with different concentrations of EGTA at pH 6.8, conditions under which microsomes were kept in the enzyme syringe in studies with Ca\textsuperscript{2+}-free microsomes, did not appreciably alter the ATPase and calcium-binding activities of the microsomes for considerable period of time (up to 2 h).

**Table I**

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Ca\textsuperscript{2+}-free microsomes</th>
<th>Ca\textsuperscript{2+}-bound microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial velocity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP formation</td>
<td>Ca binding</td>
<td>Ca/EP</td>
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<td>0.024</td>
</tr>
<tr>
<td>Phosphorylated</td>
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<td>0.060</td>
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</table>

* Calculated by dividing the rate of calcium binding by the rate of EP formation.
were almost attained. The protein concentration in the final calcium-binding assay medium was 0.43 mg/ml. The initial rates of EP formation were determined by the standard procedures. Microsomal vehicle solutions containing different amounts of EGTA. The ATPase reactions were started by the additions of 0.1 mM CaCl₂ through the rapid quenching apparatus, and the amount of EP formed was determined by the standard procedures. Microsomal protein concentration in the final calcium-binding assay medium was 0.63 mg/ml. The initial rates of EP formation (C) were estimated by determining the amounts of EP formed per 10 ms, taking the initial slopes formed by initial few points during the upstrokes of the time courses (micromoles of phosphate/g of microsomal protein-10 ms). Maximal amounts of EP formation (L) were those formed at 200 ms after starting the reaction, where the steady state of reactions were almost attained. The inset represents Ca²⁺ dependence profile of the half-time (τ, A) of EP formation taken from the same experimental data. Cardiac microsomes, preincubated with [γ-³²P]ATP and with or without cAMP-dependent protein kinase, gave phoshoester phosphorylation of 0.01 and 0.015 pmol of phosphate/g of microsomal protein, respectively. The initial rate of EP formation was increased markedly by phosphorylation of phospholamban at concentrations of Ca²⁺ ranging from 0.2 to 14 μM. While the effect of phospholamban phosphorylation on the initial rate is still evident when Ca²⁺ concentrations were 0.2 μM or lower, as was partially evidenced from the plots of τ₁/₂ against pCa (inset, Fig. 3), the levels of EP at the steady state were almost unaltered by phosphorylation at Ca²⁺ concentrations below 1 μM. At higher Ca²⁺ concentrations of 20 μM or higher the protein kinase effects on the rate and amount of EP formation were evident. In Ca²⁺-bound microsomes, the stimulatory effects of phosphorylamban phosphorylation were also seen (Fig. 1), although the accurate analyses were precluded because the reaction was more rapid and the effect of phosphorylation was less evident.

Effect of cAMP-dependent Phosphorylation on EP Formation at Different ATP Concentrations—Fig. 4 typically represents time courses of EP formation by control and phosphorylated cardiac microsomes, Cardiac microsomes (8.0 mg/ml) were preincubated under standard conditions without (open symbols) or with (closed symbols) cAMP-dependent protein kinase at 25°C for 3 min in a total volume of 6.0 ml, and were passed through the anion exchange resin after dilution with buffer solution. The resultant eluates were added to the standard vehicle solutions containing different amounts of EGTA. The ATPase reaction was started by the addition of 0.11 mM CaCl₂ and different concentrations of [γ-³²P]ATP, through the rapid quenching apparatus. Microsomal protein concentration in the final ATPase assay medium was 0.42 mg/ml. Cardiac microsomes, preincubated with [γ-³²P]ATP and with or without cAMP-dependent protein kinase, exhibited phosphoester phosphorylation of 0.64 and 0.02 pmol of phosphate/g of microsomal protein, respectively. The maximal amount of EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP). The maximal amount of EP formed (Fig. 5A) and the initial rate of EP formation (Fig. 5B) determined from data in Fig. 4 were plotted against ATP concentrations. Fig. 5A shows that the amount of maximal EP formation was lower at lower ATP concentrations (1 to 5 μM ATP).
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\(\mu M\) and higher at higher ATP concentrations (\(\geq 10 \mu M\)) in phosphorylated microsomes than in control. Fig. 5B also shows that the initial rate of EP formation by phosphorylated microsomes was markedly higher at higher ATP concentrations above 10 \(\mu M\), whereas at lower ATP concentrations the rate was lower in phosphorylated microsomes, compared with control (cf. Fig. 6). In the latter the observed initial velocity of EP formation is considered to represent merely an apparent rate since the initial rate of Pi liberation exhibited a significant increase in the presence of lower ATP concentrations (1 to 5 \(\mu M\)), where EP formation was inhibited, when microsomes were phosphorylated by cAMP-dependent protein kinase (Fig. 6; also cf. Figs. 4 and 5). The apparent inhibition of EP formation in phosphorylated microsomes is probably related to the increased rate of EP decomposition (3), which becomes more apparent at lower ATP concentrations where the rate of EP formation is lower, becoming comparable to that of EP decomposition (see "Discussion").

Effect of cAMP-dependent Phosphorylation on Calcium Binding by Cardiac Microsomes—Fig. 7 shows the Ca\(^{2+}\) dependence profile of the initial rate of \(^{45}\)Ca binding by control and phosphorylated microsomes in the assay system in which Ca\(^{2+}\)-free microsomes were reacted with 10 \(\mu M\) ATP and various concentrations of \(^{45}\)Ca\(^{2+}\). The initial rates and maximal amounts of calcium binding were dependent upon Ca\(^{2+}\) concentrations between 0.2 and 14 \(\mu M\), and exhibited marked increases after cAMP-dependent phosphorylation of phospholamban. The effects were evident even when Ca\(^{2+}\) concentrations were 0.1 \(\mu M\) or lower. In accord with findings in the EP

![Fig. 6. Effect of cAMP-dependent phosphorylation on the rate of Pi liberation and EP formation in Ca\(^{2+}\)-free microsomes. Cardiac microsomes (7.8 mg/ml) were preincubated under standard conditions without (open symbols) and with (closed symbols) CAMP-dependent protein kinase (4.0 mg/ml) at 25°C for 2 min in a total volume of 6.0 ml, and were passed through the anion exchange resin after dilution with buffer solution. The resultant eluates were added to the standard vehicle solution containing different concentrations of EGTA. The calcium-binding reactions were started by the additions of 10 \(\mu M\) ATP and 0.1 mM \(^{45}\)CaCl\(_2\) through the rapid quenching apparatus, and the amounts of \(^{45}\)Ca bound were determined as described under "Experimental Procedures." The initial rate of calcium binding (\(v_0\)) was determined by taking the initial slope (micromoles of \(^{45}\)Ca/g of microsomal protein-10 ms\(^{-1}\)), formed by initial few points during the upstroke of the calcium binding curve, and the maximal amounts of calcium binding (\(v_\infty\)) were those at 200 ms, where the steady states of the reactions were attained. Microsomal protein concentration in the final calcium-binding assay medium was 0.56 mg/ml. Cardiac microsomes, preincubated with [\(^{32}\)P]ATP and with and without CAMP-dependent protein kinase, exhibited phosphoester phosphorylation of 0.62 and 0.02 \(\mu mol\) of phosphate/g of microsomal protein, respectively.

From the initial phase of time course of calcium-binding studies performed under conditions identical with Fig. 7, the half-time (\(t_{1/2}\)) to attain the maximal amount of calcium binding was estimated at five different Ca\(^{2+}\) concentrations.

\[
\begin{array}{cccc}
\text{Microsomes} & \text{Half-time \((t_{1/2})\) of maximal calcium binding at Ca\(^{2+}\) concentration of} & \text{Mean} \pm \text{S.E.}\n\hline
& \text{ms} & \text{ms} \\
\text{Control} & 37 & 38 & 36 & 40 & 37 & 37.6 \pm 1.4 \\
\text{Phosphorylated} & 20 & 22 & 18 & 17 & 17 & 18.8 \pm 1.9 \\
\end{array}
\]

\(t_{1/2}\) values of calcium binding was greatly shortened by cAMP-dependent phosphorylation of phospholamban (Table II), the \(t_{1/2}\) values being almost independent of Ca\(^{2+}\) concentrations within a wide range between 0.2 and 14 \(\mu M\).
The present study was aimed at examining the effects of cAMP-dependent protein kinase-catalyzed phosphorylation of phospholamban on the transient state kinetic properties of cardiac sarcoplasmic reticulum, since the phosphorylated state of phospholamban was previously shown to be related to a marked increase in the overall rates of ATP hydrolysis and calcium uptake (4, 6-16) through an enhancement of the elementary step(s) of Ca2+-dependent ATPase enzyme of cardiac sarcoplasmic reticulum (3, 6, 7). In view of the extreme rapidity at which the ATPase enzyme reacts with ATP and calcium (19-23), a chemical quench flow device (24) was used to follow the transient time courses of EP formation, P liberation, and calcium binding by cardiac sarcoplasmic reticulum with time intervals as short as 5 ms. It was found that cAMP-dependent phosphorylation of phospholamban results in marked increases in both the initial rates and maximal extents of EP formation and calcium binding. The effects were more evident when cardiac microsomes pretreated with EGTA to remove Ca2+ bound to the enzyme were allowed to react with Ca2+ and ATP. Thus, under such conditions the initial velocities of EP formation and calcium binding were stimulated more than 2-fold within the physiological range of Ca2+ (0.1 to 20 μM) and at ATP concentrations above 10 μM, when cAMP-dependent protein kinase catalyzed phosphorylation of phospholamban. It was also found that the stoichiometric coupling between EP formed and calcium transported is well maintained at 1:2 under these conditions, in accord with the thermodynamic analysis of this system (19, 32).

Observed alterations in the enzymatic activity produced by phospholamban phosphorylation are probably brought out by increases in both the rate at which calcium ions and ATP bind to the ATPase enzyme and the rate at which EP is subsequently formed in these membranes, i.e. either or all of Steps i, ii, and iii in the following equation:

\[
E + 2Ca^{2+} \rightarrow E.Ca^{2+} \rightarrow E.Ca^{2+}.ADP^{\prime} \rightarrow E.Ca^{2+}.P_{\cdot} \rightarrow E + P_{\cdot} + 2Ca^{2+}
\]

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 Ca2+ and 1 mol of ATP bind 1 mol of E to form the Michaelis complex \(E.Ca^{2+}\). This is followed immediately by the formation of the phosphorylated intermediate \(E.P_{\cdot}\) (referred to as EP throughout the present report) 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 \(P_{\cdot}\). Among these intermediary steps, Step iv is considered to be rate-determining in the presence of saturating concentrations of ATP and Ca2+, whereas Step ii is not the rate-determining step unless the ATP concentration is extremely lowered (19). In the present study the overall rate of EP formation (Steps i, ii, and iii) was determined by measuring the initial rate of EP formation during the transient time course, after microsomes were incubated with appropriate concentrations of calcium and ATP, while the apparent initial rate of calcium binding (Step i) was assessed to some extent by determining the rates of calcium binding and EP formation after Ca2+-free and Ca2+-bound microsomes were incubated with calcium and ATP.

The apparent initial rate of calcium binding, coupled with EP formation, determined from incubation of Ca2+-free microsomes with calcium and ATP exhibited a marked increase after cAMP-dependent phosphorylation of phospholamban (Figs. 1 and 2). Such an increase was evident within a wide range of Ca2+ concentrations and as low as 0.1 μM Ca2+ in experiments on both calcium binding (Fig. 7) and EP formation (Fig. 3). The apparent half times \((t_{1/2})\) of calcium binding (Table II) and EP formation (inset, Fig. 3), which were virtually independent of Ca2+ concentration, also exhibited marked shortening when phospholamban was phosphorylated, although the latter assessment does not represent the true initial rates (see above). These results indicate that the apparent initial rate of calcium binding by the ATPase enzyme (mainly Step i in Equation 1) is markedly enhanced by cAMP-dependent phosphorylation of phospholamban. It was also indicated from these studies and those on the ATP concentration dependence (Figs. 4 and 5) that the rate at which ATP that Ca2+-dependent ATPase from skeletal and cardiac muscle microsomes were mainly carried out employing EGTA-treated microsomes as it turns over during the translocation of Ca2+. Therefore, the rate-limiting step would be that at which calcium binds to \(E\). Ca2+ formation as follows:

\[
E.Ca^{2+} \rightarrow E + Ca^{2+} + 2ATP
\]

where \(1\) is markedly enhanced by CAMP-dependent phosphorylation of phospholamban. The possibility that the observed EP formation represents that of \(E-P\), a major portion of the reaction intermediate (19), which is supposed to react with ADP to form ATP, was indicated by the experiments in which the sensitivity of EP to ADP was examined; virtually all of the EP in control and phosphorylated microsomes was immediately decomposed when ADP was added to EP simultaneously with or shortly after addition of EGTA.

The present studies on the effect of cAMP-dependent phosphorylation were mainly carried out employing EGTA-treated microsomes (Ca2+-free enzymes) in which the effects of protein kinase on the initial rates were more evident and more readily detectable. However, in view of the observations made by Sumida et al. (23) that in Ca2+-bound microsomes the initial amount of transported calcium (at time zero) represents the calcium that was bound to the vesicles before ATP had been added and that the additional calcium would be transported at the rate essentially compatible with that of the Ca2+-free microsomes, the protein kinase-induced alterations in enzymatic parameters similar to what were found in Ca2+-free microsomes described above would also occur in Ca2+-bound microsomes as it turns over during the translocation of Ca2+. Indeed, we found an increase in the rates and amounts of EP formation (Fig. 1) and calcium binding (Fig. 2) in Ca2+-bound microsomes after phosphorylation of phospholamban, although the effects were obscured by the rapidity of the initial rate in the former and the significant amount of calcium initially bound by the enzyme in the latter.

\[
F \stackrel{(i)}{\rightarrow} E + 2Ca^{2+} \rightarrow E.Ca^{2+} \rightarrow E.Ca^{2+}.ADP^{\prime} \rightarrow E.Ca^{2+}.P_{\cdot} \rightarrow E + P_{\cdot} + 2Ca^{2+}
\]

(2)

Therefore, the rate-limiting step would be that at which \(E\) is converted to \(E\) (Step i), rather than that at which calcium binds to \(E\) (Step ii). Thus, it is possible to assume that one of the major steps that is altered by protein kinase-induced phosphorylation is Step i. Taking these into consideration, we refer this step as to the apparent initial step of calcium binding.

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Therefore, the rate-limiting step would be that at which \(E\) is converted to \(E\) (Step i), rather than that at which calcium binds to \(E\) (Step ii). Thus, it is possible to assume that one of the major steps that is altered by protein kinase-induced phosphorylation is Step i. Taking these into consideration, we refer this step as to the apparent initial step of calcium binding.

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Protein Kinase Effects on Transient Kinetics of Ca²⁺-ATPase

The present demonstration that the initial rate and maximal amount of EP formation in phosphorylated microsomes were lower at lower ATP concentration ranges (Figs. 4 and 5) is compatible with our previous observations (3) that the rate of EP decomposition (Step iv in Equation 1) is greatly enhanced by cAMP-dependent phosphorylation of phospholamban. Such enhancement is presumably more effective in reducing the apparent velocity and maximal amount of EP formation at lower ATP concentration ranges (Figs. 4 and 5), where the EP formation (Step ii, or iii, or both) is slower and comparable to the EP decomposition (Step iv), whereas the initial velocity and maximal amount of EP formation were greatly augmented in phosphorylated microsomes at higher ATP concentration ranges (Figs. 4 and 5), where the EP formation occurs much faster than the EP decomposition. In fact, cAMP-dependent phosphorylation of phospholamban resulted in the significant increases in the initial rates of Pᵢ liberation at lower ATP concentration ranges (Fig. 6), while the rate and amount of EP formation remained lower at these concentrations of ATP, suggesting that the observed reduction in EP formation at lower ATP concentrations in phosphorylated microsomes can be attributed to the predominance of a protein kinase-induced increase in the EP decomposition at lower ATP concentration ranges which becomes manifest due to relatively reduced rate of EP formation.

The present observations that the initial rates of EP formation and calcium binding by cardiac microsomes are markedly enhanced by protein kinase-catalyzed phosphorylation with the maintenance of the stoichiometric coupling of 2 mol of calcium transported/mol of EP formed, together with our previous report (3) that the rate of EP decomposition is also enhanced by phosphorylation, are compatible with the accumulated body of evidence that the overall rates of production of Pᵢ (3, 9, 16) and ADPᵢ (3) and accumulation of Ca⁴⁺ (4, 8-15) are greatly enhanced by protein kinase-catalyzed phosphorylation of a microsomal protein of 20,000 to 22,000 daltons (phospholamban), and thus suggest that the ATPase enzyme itself, but not the efficiency of coupling, is directly affected by protein kinase-catalyzed phosphorylation of phospholamban. The increase in the pseudo steady state rates of calcium binding, coupled with the elementary steps of ATPase, produced by phospholamban phosphorylation, may largely account for the previous findings that the stimulatory effects of protein kinase on the overall rate of ATP hydrolysis and calcium uptake is more evident at lower Ca⁺⁺ concentration ranges (3, 9, 33).

Thus, it was indicated that the probable sites of action of phospholamban may be those steps associated with calcium and ATP binding to the enzyme and a step at which the reaction intermediate EP is subsequently formed (present study); the rate at which EP is decomposed is also enhanced (3), which can be a predominant factor in affecting the transient kinetics at lower ATP concentration ranges. Such effects produced by protein kinase can increase the apparent affinity of the Ca²⁺-dependent ATPase system for Ca²⁺⁺, resulting in the marked stimulation of the turnover rates of Ca²⁺⁺-dependent ATPase and active calcium transport by cardiac sarcoplasmic reticulum. These observations thus lent support for the hypothetical view that phospholamban can serve as a regulator controlling the active calcium transport by cardiac sarcoplasmic reticulum (3-7).

It is of interest to consider the molecular mechanism by which the ATPase enzyme of cardiac microsomes could be controlled by cAMP-dependent phosphorylation of phospholamban. Although the preliminary results are suggestive of the possibility that these effects are induced by the direct molecular interactions between the ATPase and phospholamban (7), those could also be caused by an indirect effect such as that involving the lipido-protein interactions within the membrane. If the direct protein-protein interactions are functional, phospholamban which is presumably located at the outer surface of the membrane (4, 7) could exhibit an interaction with the ATPase in a manner in which the rate of turnover of the ATPase enzyme is greatly altered as it recognizes and translocates calcium, depending upon phosphorylated and unphosphorylated states of phospholamban. It would still be premature to speculate further the relationship between the enzyme states of ATPase and the states of phospholamban, since no detailed information on the structure-function relationship between the ATPase enzyme and phospholamban is available at the present time. It would also be intriguing to consider the possible involvement of other potent factors like calcium-binding regulatory protein (34) in these systems. Possible functional relationship between these key components in manifestation of the observed control mechanism should be clarified better by purifying these and reconstructing the functioning membrane vesicles from such components and defined phospholipids.

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Protein Kinase Effects on Transient Kinetics of Ca\textsuperscript{2+}-ATPase


Transient state kinetic studies of Ca2+-dependent ATPase and calcium transport by cardiac sarcoplasmic reticulum. Effect of cyclic AMP-dependent protein kinase-catalyzed phosphorylation of phospholamban.

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