Mechanism of the Stimulation of Calcium Ion Dependent Adenosine Triphosphatase of Cardiac Sarcoplasmic Reticulum by Adenosine 3′,5′-Monophosphate Dependent Protein Kinase†

Evangelia Galani Kranias,* Frederic Mandel, Taitzer Wang, and Arnold Schwartz

ABSTRACT: Canine cardiac sarcoplasmic reticulum (SR) is known to be phosphorylated by adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase on a 22 000-dalton protein. Phosphorylation enhances the initial rate of Ca^{2+} uptake and Ca^{2+}-ATPase activity. To determine the molecular mechanism by which phosphorylation regulates the calcium pump in SR, we examined the effect of cAMP-dependent protein kinase on the individual steps of the Ca^{2+}-ATPase reaction sequence. Cardiac sarcoplasmic reticulum was preincubated with cAMP and cAMP-dependent protein kinase in the presence (phosphorylated SR) and absence (control) of adenosine 5′-triphosphate (ATP). Control and phosphorylated SR were subsequently assayed for formation (4–200 ms) and decomposition (0–73 ms) of the acid-stable phosphorylated enzyme (E∼P) of Ca^{2+}-ATPase in media containing 100 μM [ATP] and various free [Ca^{2+}]. cAMP-dependent phosphorylation of SR resulted in pronounced stimulation of initial rates and levels of E∼P formed at low free [Ca^{2+}] (≤7 μM), but the effect was less at high free Ca^{2+} (≥10 μM). This stimulation was associated with a decrease in the dissociation constant for Ca^{2+} binding and a possible increase in Ca^{2+} sites. The observed rate constant for E∼P formation of calcium-preincubated SR was not significantly altered by phosphorylation. Phosphorylation also increased the initial rate of E∼P decomposition. These findings indicate that phosphorylation of cardiac SR by cAMP-dependent protein kinase regulates several steps in the Ca^{2+}-ATPase reaction sequence which result in an overall stimulation of the calcium pump observed at steady state.

The stimulating effects of catecholamines on the relaxation of mammalian myocardium can be attributed in part to a cAMP[1]-mediated increase in the rate of calcium transport into the cardiac sarcoplasmic reticulum (LaRaia & Morkin, 1974; Kirchberger et al., 1974; Schwartz et al., 1976; Will et al., 1976). The stimulation of calcium transport may be associated with phosphorylation of a 22 000-dalton, membrane-bound protein referred to as phospholamban by Kirchberger et al. (1975). It has been known that phospholamban can be phosphorylated by both endogenous, membrane-bound (LaRaia & Morkin, 1974; Wray & Gray, 1977) and exogenous, soluble (Tada et al., 1975; Kirchberger & Tada, 1976; Schwartz et al., 1976; Will et al., 1976) cAMP-dependent protein kinases (EC 2.7.1.37, ATP:protein phosphotransferase).

It is generally accepted that the membrane-bound Ca^{2+},-Mg^{2+}-ATPase (EC 3.6.1.3, ATP phosphohydrolase) is responsible for the active transport of calcium into the SR lumen. The reaction sequence of this enzyme is often presented as shown in eq 1 (Froehlich & Taylor, 1975).

$$E' \overset{+2Ca^{2+}}{\underset{(1)}{\leftrightarrow}} E{\cdot}Ca_2 \overset{ATP}{\underset{(2)}{\leftrightarrow}} E{\cdot}Ca_2{\cdot}ATP \underset{(3)}{\leftrightarrow} ADP + Ca_2{\cdot}E{\sim}P \underset{(4)}{\leftrightarrow} Ca_2E{\cdot}P \overset{-2Ca^{2+}}{\underset{(5)}{\leftrightarrow}} E' + P_i \quad (1)$$

cAMP-dependent phosphorylation of SR membranes has been shown to correlate well with cAMP-induced stimulation of steady-state Ca^{2+}-ATPase activities (Tada et al., 1974; Wray & Gray, 1977). Recently, the effect of phosphorylation on the transient state Ca^{2+}-ATPase activity has been studied by using a quench-flow apparatus with a reported resolution time of 50 ms (Tada et al., 1979). The authors, using 500 ms as their first measured point, reported that cAMP-dependent phosphorylation of cardiac SR lowered the E∼P levels at free Ca^{2+} concentrations of 0.1–10 μM and enhanced the rate of E∼P decomposition but did not observe any change in the rate of formation of E∼P. Studying the Ca^{2+}-ATPase activity in the 0–200-ms time range, we report that phosphorylation of the SR membranes results in a marked stimulation of both the initial rate and the steady-state levels of E∼P at concentrations of free Ca^{2+} of 0.2–7 μM. At higher free Ca^{2+} concentrations (≥10 μM), a much smaller stimulation was observed. Also, phosphorylation of the SR increases the initial rate of E∼P decomposition (0–73 ms).

Experimental Procedures

Materials

All biochemical reagents including beef heart cAMP-dependent protein kinase were purchased from Sigma Chemical Co. All chemicals were of "chemical pure grade". Na_2ATP was purchased from Boehringer Mannheim and [γ-^{32}P]ATP, ammonium salt (10–40 Ci/mmol), was purchased from New England Nuclear.

Methods

Miscellaneous Methods. Sodium dodecyl sulfate (0.1%)–polyacrylamide (7.5%) gel electrophoresis of ^{32}P-labeled sarcoplasmic reticulum, isolated by Sephadex G-50 column chromatography, was carried out at pH 7.1 according to the methods of Maizel (1969), Weber & Osborn (1969), and Laemli (1970). The molecular weight of the radioactive peak was determined with the aid of a relative mobility curve composed of appropriate standard markers, phosphorylase *a*

† From the Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267. *Received April 2, 1980.* The work was supported by U.S. Public Health Service Grant P01 HL 22619-02 (IV A) and by a Grant-in-Aid from the Southwestern Ohio Chapter of the American Heart Association.

[1] Abbreviations used: SR, sarcoplasmic reticulum; cAMP, adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; M_r, molecular weight; EDTA, ethylenediaminetetraacetic acid.

0006-2960/80/0419-5434$01.00/0

(M_r 100 000), human serum albumin (M_r 66 000), DNase 1 (M_r 31 000), and egg white lysozyme (M_r 14 300). Calcium–EGTA buffers contained 0.1 mM (final concentration) EGTA and various concentrations of $CaCl_2$. Free calcium concentrations at pH 6.8 were calculated by using the association constants of Sillen & Martell (1964). A computer program was used to calculate the total concentration of calcium required to obtain various free calcium concentrations at pH 6.8 in the presence of 3 mM total Mg and 10 or 100 μM ATP.

Preparation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum (SR) from dog cardiac muscle was prepared as previously described (Sumida et al., 1978). The preparation was stored at 0 °C in 20 mM Tris–maleate buffer (pH 6.8) containing 100 mM KCl and was used within 20 h. The final yield was 1 mg of SR protein/g of wet cardiac tissue. The SR preparations contained cAMP-dependent protein kinase activity (Kranias et al., 1980). The purity and homogeneity of the preparations was checked by electron microscopy after negative staining, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and by various enzyme marker activities. Sarcolemmal and mitochondrial contaminations were low, as judged from electron micrographs and from 5′-nucleotidase (2.6 ± 0.9 mol mg^{-1} h^{-1}), [^{3}H]ouabain binding (4–10 pmol/mg), and cytochrome *c* oxidase (0.35 μmol mg^{-1} h^{-1}) activities. The SR Ca^{2+},Mg^{2+}-ATPase (50 μg of protein/mL) was assayed in 25 mM histidine buffer (pH 7.0) containing 5 mM $MgCl_2$, 100 mM KCl, 5 mM NaN_3, 100 μM EGTA, 100 μM $CaCl_2$ (10 μM free Ca^{2+}), and 5 mM ATP; the specific activity was 40–60 μmol of P_i mg^{-1} h^{-1} at 37 °C. The rate of Ca^{2+} uptake was determined at 20 °C in 40 mM Tris–maleate buffer (pH 6.8) containing 100 mM KCl, 10 mM $MgCl_2$, 5 mM NaN_3, 5 mM Tris–oxalate, 5 mM ATP, 100 μM EGTA, and 100 μM $CaCl_2$ (10 μM free Ca^{2+}) and was found to be 20–25 μmol mg^{-1} h^{-1}.

Phosphorylation of Cardiac Sarcoplasmic Reticulum. Phosphorylation was carried out at 30 °C in 50 mM phosphate buffer (pH 6.8) containing 10 mM $MgCl_2$, 10 mM NaF, 1 mg/mL sarcoplasmic reticulum vesicles, and 500 μM ATP (including [γ-^{32}P]ATP). The final concentrations of cAMP and cAMP-dependent protein kinase, when added, were 1.0 μM and 50 μg or 100 pM, respectively, per mL of reaction mixture. The reaction was terminated by the addition of 2 mL of 7% ice-cold perchloric acid containing 7% polyphosphate and 0.5 mg of carrier skeletal sarcoplasmic reticulum protein. The samples were centrifuged and the pellet was washed 3 times with 7% perchloric acid containing 7% polyphosphoric acid. The final pellet was dissolved in 0.5 mL of 10 mM NaOH containing 0.1 mM Na_3PO_4, and radioactivity of ^{32}P was determined in 10 mL of Aquasol II (New England Nuclear) in a Model 3320 Packard Tri-Carb liquid scintillation spectrometer.

In experiments in which the effect of protein kinase catalyzed phosphorylation of E∼P formation and decomposition was studied, the SR vesicles were preincubated in the same reaction medium described above except that unlabeled ATP was used and sodium fluoride was omitted. The extent of SR phosphorylation in the absence of NaF was lower by 30%. Control vesicles were also incubated under identical conditions without ATP. After 5 min of incubation at 30 °C, the mixture was centrifuged at 105000*g* for 30 min and washed twice, and the pellet was homogenized gently in ice-cold 20 mM Tris–maleate buffer (6.8) containing 100 mM KCl. The recovery of phosphorylated and control sarcoplasmic reticulum protein after this procedure was ∼85%. The phosphoester bonds due to the protein kinase mediated phosphorylation of SR were found to be stable during centrifugation and washing of the phosphorylated SR.

Transient-State Kinetic Studies. Rapid mixing experiments were performed as previously described (Sumida et al., 1978) by using a chemical quench-flow apparatus. The standard vehicle solution for the SR, substrate, and EGTA was a 20 mM Tris–maleate buffer (pH 6.8) containing 0.1 M KCl, 3 mM $MgCl_2$, and 5 mM NaN_3. The temperature was controlled at 20 °C by a constant temperature circulator.

Formation of Phosphorylated Intermediate of Ca^{2+}-ATPase. In these experiments the enzyme syringe contained canine cardiac SR vesicles (1–2.5 mg/mL) in the standard vehicle solution including 100 μM EGTA and various $CaCl_2$ concentrations (free Ca^{2+} concentration of 0.1–10 μM). The substrate syringe contained 200 μM ATP (including [γ-^{32}P]ATP), 100 μM EGTA, and various $CaCl_2$ concentrations (0.1–10 μM Ca^{2+}) in the standard vehicle solution. The perchloric acid syringe contained 9% perchloric acid and 20% polyphosphate. The time course of formation of the acid-stable phosphorylated enzyme was measured from 4.3 to 200 ms. The zero-time blank prepared by mixing the enzyme with acid followed by substrate was subtracted from each sample.

Decomposition of Phosphorylated Intermediate of Ca^{2+}-ATPase. In these experiments the rate of decomposition of the acid-stable intermediate of the Ca^{2+}-ATPase was directly measured at 20 °C in the presence of 2 mM EGTA. The enzyme syringe contained cardiac SR vesicles (1–1.5 mg/mL) in the standard vehicle solution including 100 μM EGTA and 100 μM $CaCl_2$ (10 μM free Ca^{2+}). The substrate syringe contained 200 μM ATP (including [γ-^{32}P]ATP), 100 μM EGTA, and 100 μM $CaCl_2$ in the standard vehicle solution. The EGTA syringe contained 6 mM EGTA in the standard vehicle solution. The perchloric acid syringe contained 9% perchloric acid and 20% polyphosphate. Formation of the phosphorylated Ca^{2+}-ATPase intermediate (E∼P) was allowed to proceed for 116 ms. Further formation of E∼P was terminated by chelating the free calcium with 2 mM (final) EGTA, and the level of E∼P was taken as the zero-time E∼P level ($[E\sim P]_{max}$). Subsequent addition of perchloric acid to quench the E∼P decomposition allowed measurement of the decreasing E∼P level at various times, ranging from 3.6 to 73 ms. The rate of E∼P decay was determined by plotting log $[E\sim P]_t/[E\sim P]_{max}$ vs. time.

Determination of Dissociation Constant for SR·Calcium Complex. An ATP (200 μM) solution containing EGTA (4 mM) was mixed with an equal volume of SR preincubated with Ca–EGTA buffer, which gave various concentrations of free Ca^{2+} (final 1–8 μM). The reaction was quenched with perchloric acid as described above. The purpose was to determine the $[E{\cdot}Ca_2]$ concentration by indirectly measuring the time course of initial E∼P formation. Under such reaction conditions, EGTA chelates free Ca^{2+} to prevent the formation of new $E{\cdot}Ca_2$ during the course of phosphorylation. The dissociation rate constants for $E{\cdot}Ca_2$ (9 s^{-1}) and E∼P (9 s^{-1}) are small compared with the apparent, pseudo-first-order rate constant for E∼P formation (116–173 s^{-1}) [see Sumida et al. (1978, 1980) and Figure 2]; therefore the reaction can be expressed as

$$E{\cdot}Ca_2 \xrightarrow{k[ATP]} E\sim P \qquad (2)$$

The rate equation for E∼P is

$$[E\sim P] = [E{\cdot}Ca_2](1 - e^{-k[ATP]t}) \qquad (3)$$

from which $[E{\cdot}Ca_2]$ and k[ATP] (or k at 100 μM ATP) can be estimated by a nonlinear regression program. The values

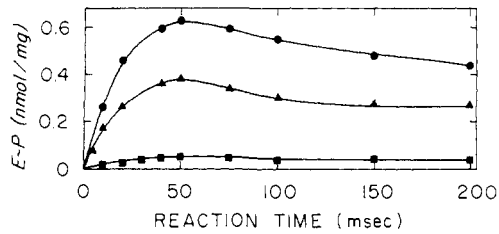

FIGURE 1: Time courses of the formation of phosphorylated enzyme, E∼P, of calcium-preloaded cardiac SR. SR was preincubated with 1 μM cAMP, 10 mM $MgCl_2$, and 50 mM phosphate buffer (pH 6.8) (●) in the presence of 10 mM NaF (■) or 2 mM EDTA (▲) for 5 min at 30 °C. SR was then centrifuged, washed twice, and assayed for E∼P formation as described under Methods. Reaction conditions were as follows: SR, 0.7 mg/mL; ATP, 10 μM; EGTA, 100 μM; $CaCl_2$, 100 μM (10 μM free Ca^{2+}); $MgCl_2$, 3 mM; KCl, 0.1 M; NaN_3, 5 mM; Tris–maleate, 20 mM, pH 6.8; 20 °C.

of $[E{\cdot}Ca_2]$ obtained at various concentrations of free Ca^{2+} were then used in the determination of K_d by double-reciprocal plots of $[E{\cdot}Ca_2]$ vs. $[Ca^{2+}]^2$

$$E + 2Ca^{2+} \overset{K_d}{\rightleftharpoons} E{\cdot}Ca_2$$

$$\frac{1}{[E{\cdot}Ca_2]} = \left(\frac{K_d}{[E{\cdot}Ca_2]_{max}}\right)\left(\frac{1}{[Ca^{2+}]^2}\right) + \frac{1}{[E{\cdot}Ca_2]_{max}} \quad (4)$$

Results

cAMP-Dependent Phosphorylation of Cardiac SR. Cardiac SR, prepared as described under Methods, was incubated in the presence of cAMP or in the presence of cAMP and cAMP-dependent protein kinase. The product of phosphorylation by endogenous or exogenous cAMP-dependent protein kinase was not extracted with chloroform–methanol (2:1 v/v) or by hot trichloroacetic acid, ruling out lipid or nucleic acid as the phosphorylated component. The phosphorylated SR was hydrolyzed by hot 0.5 N NaOH, but it was resistant to cleavage by 0.8 M hydroxylamine indicating the presence of phosphoester bonds. Phosphorylated cardiac SR, isolated by Sephadex G-50, was subjected to gel electrophoresis under denaturing conditions, and a 22000 M_r protein was identified as the only ^{32}P-acceptor protein (Kranias et al., 1980). These results are in agreement with previous observations (LaRaia & Morkin, 1974; Kirchberger & Tada, 1976; Schwartz et al., 1976; Wray & Gray, 1977). Phosphorylation of the 22000 M_r protein was abolished in the presence of the heat-stable protein kinase inhibitor isolated from rabbit skeletal muscle. Phosphate incorporation into the 22000 M_r protein varied with different SR preparations. The average value for twenty preparations was 0.5 ± 0.2 nmol of P_i/mg of SR protein and 1.5 ± 0.5 nmol of P_i/mg of SR protein by the endogenous and exogenous protein kinases, respectively. Phosphorylation of cardiac SR by cAMP-dependent protein kinase was not Ca^{2+} activated. Furthermore, the addition of 10 μM free Ca^{2+} inhibited the cAMP-dependent phosphorylation of cardiac SR, in agreement with the previous observations of LaRaia & Morkin 1974) and Wray & Gray (1977).

Transient-State Ca^{2+}-ATPase Activity of SR Phosphorylated by Protein Kinase. Cardiac SR was preincubated under each of the conditions used for phosphorylation of SR as described under Methods. The reactants (phosphate buffer, $MgCl_2$, and cAMP), temperature, and time of incubation of the phosphorylation assay as well as subsequent washing of the SR had no effect on the rates of formation and decomposition of the phosphorylated intermediate of Ca^{2+}-ATPase (E∼P). The presence of sodium fluoride in the preincubation medium stimulated phosphorylation of SR, but it resulted in

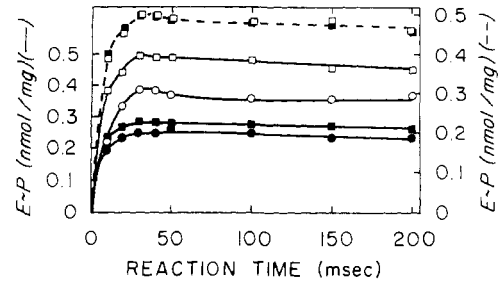

FIGURE 2: Effect of cAMP-dependent phosphorylation of cardiac SR on the time course of formation of the phosphorylated enzyme intermediate, E∼P, of Ca^{2+}-ATPase. Cardiac SR (1 mg/mL) was preincubated with 1 μM cAMP and 0.1 mg/mL cAMP-dependent protein kinase in 10 mM $MgCl_2$ and 50 mM phosphate buffer (pH 6.8) in the presence (open symbols) or absence (closed symbols) of 500 μM ATP, for 5 min at 30 °C. Reaction mixtures were centrifuged, and SR's were washed twice and resuspended in 20 mM Tris–maleate buffer (pH 6.8) containing 100 mM KCl. ATPase reaction was carried out as described under Methods. Reaction conditions were as follows: SR, 0.8 mg/mL; ATP, 100 μM; EGTA, 100 μM; $CaCl_2$, 76 μM (2 μM free Ca^{2+}) (●, ○) or 88 μM (4 μM free Ca^{2+}) (■, □). The *dotted line* is the normalized curve for control and phosphorylated SR assayed at 4 μM free Ca^{2+}.

an irreversible inhibition (80–90%) of the levels of E∼P formed (Figure 1). Also, unlike previous observations preincubation in the presence of 0.5 mM EGTA or 2 mM EDTA similar to the control conditions by Tada et al. (1979) resulted in a 40% decrease in the levels of E∼P formed at pH 6.8 (Figure 1). Therefore, for the transient-state kinetic studies, control cardiac SR was preincubated under identical conditions with those for phosphorylated SR but in the absence of ATP. Control and phosphorylated SR were processed simultaneously and were assayed within 30 min of each other. The rate and levels of E∼P formed and the rate of E∼P decomposition for control SR were not significantly different from those of the same SR preparation which was not subject to preincubation. The phosphoester bond formed during preincubation of the cardiac SR with the protein kinase was stable through the two washes of the phosphorylated SR. The amounts of ATP and P_i remaining with the phosphorylated cardiac SR after two washes were <0.2% of the [ATP] (100 μM) added to the reaction medium for E∼P formation.

Effect of Phosphorylation by cAMP-Dependent Protein Kinase on Formation of Phosphorylated Intermediate (E∼P) of Ca^{2+}-ATPase. Formation of the phosphorylated intermediate of Ca^{2+}-ATPase was measured from 10 to 200 ms. Phosphorylation of cardiac SR by protein kinase resulted in stimulation of both the apparent initial rate of E∼P formation and the steady-state level of E∼P (Figure 2). The maximal (at 30 ms) level of E∼P formed at 4 μM free Ca^{2+} and 100 μM ATP is 0.28 and 0.5 nmol/mg of SR for control and phosphorylated cardiac SR, respectively (Figure 2). The dotted line in the same figure is the normalized curve (the control curve normalized to the phosphorylated E∼P level at 30 ms by multiplying by a factor of 1.76) showing the same half-time (5–6 ms) for approach to maximal E∼P level for control and phosphorylated SR vesicles assayed at 4 μM free Ca^{2+}. Phosphorylation of cardiac SR resulted in stimulation of the steady-state (100 ms) levels of E∼P at $[Ca^{2+}]$ up to 7 μM. This effect of phosphorylation was not apparent when E∼P formation was assayed at higher free calcium concentrations (≥10 μM) in the presence of ATP at either high (100 μM) or low (10 μM) concentrations (Figure 3). The lack of an effect of cAMP-dependent phosphorylation on E∼P formation assayed at 10 μM free Ca^{2+} was independent of the amount of phosphoester formed in the preincubation assay.

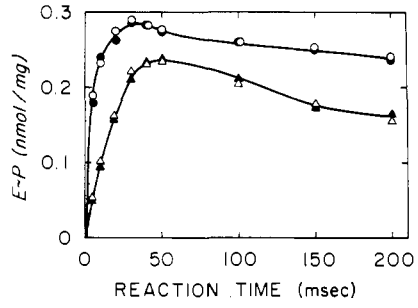

FIGURE 3: Effect of cAMP-dependent phosphorylation of cardiac SR on the time course of formation of the phosphorylated enzyme intermediate, E~P, of the Ca^{2+}-ATPase. Cardiac SR was preincubated in the presence (closed symbols) or absence (closed symbols) of ATP and subsequently assayed for E~P formation as described in Figure 2. Reaction conditions were as follows: SR, 0.5 mg/mL; EGTA, 100 μM; $CaCl_2$, 100 μM (10 μM free Ca^{2+}); ATP, 10 μM (△, ▲) or 100 μM (○, ●).

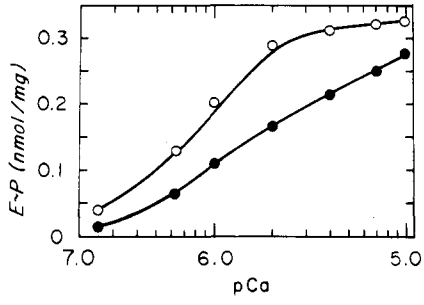

FIGURE 4: Effect of cAMP-dependent phosphorylation of cardiac SR on the Ca^{2+}-dependence profile of E~P levels at 4.3 ms. Cardiac SR was preincubated in the presence (open symbols) and absence (closed symbols) of ATP as described in Figure 2. ATPase reactions were carried out as described under Methods. SR, 0.7 mg/mL; ATP, 100 μM.

Effect of Phosphorylation by cAMP-Dependent Protein Kinase on Initial Rate of E~P Formation. cAMP-dependent phosphorylation of cardiac SR resulted in stimulation of the E~P levels even in the initial phase (4.3–10 ms) of the reaction at various free $[Ca^{2+}]$. The calcium-dependence profile of the E~P levels at 4.3 ms, the first time point obtained in these studies, is shown in Figure 4. The E~P levels were higher in phosphorylated than in control SR within a range of ionized calcium between 0.1 and 10 μM (Figure 4).

The E~P levels were also higher in phosphorylated than in control SR when assayed under conditions (in the presence of 2 mM EGTA) which prevented new $E{\cdot}Ca_2$ formation. In Figure 5 the initial time course of E~P formation is shown for control and phosphorylated cardiac SR, assayed at 1, 4, and 8 μM free $[Ca^{2+}]$. The inset in Figure 5 shows the normalized curve for control and phosphorylated SR with respect to E~P levels obtained at 4.3 ms and assayed at 8 μM free Ca^{2+} (the control and phosphorylated curves are multiplied by 2.32 and 1.73, respectively, at 1 μM free Ca^{2+} and 1.12 and 1.18, respectively, at 4 μM free Ca^{2+}). The normalized data show the same pattern for E~P formation in control and phosphorylated SR assayed at various free $[Ca^{2+}]$. Such data were fit directly with a nonlinear regression program, and the values for k and $[E{\cdot}Ca_2]$ were obtained as described under Methods. The rate constant k was found to be 269 ± 14 s^{-1} for control and 278 ± 27 s^{-1} for phosphorylated cardiac SR (n = 4), indicating that phosphorylation of SR by protein kinase does not significantly alter this rate constant. Double-reciprocal plots of $[E{\cdot}Ca_2]$ vs. $[Ca^{2+}]^2$ show that the approximate K_d for two calciums is significantly lower for phosphorylated (0.33 μM^2) than for control (1.3 μM^2) SR

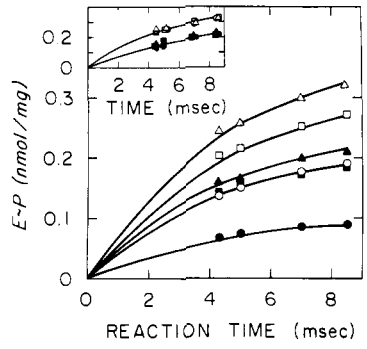

FIGURE 5: Effect of cAMP-dependent phosphorylation of cardiac SR on velocity of E~P formation. Cardiac SR preincubated in the presence (open symbols) and absence (closed symbols) of ATP was assayed for E~P formation as described under Methods. Reaction conditions were as follows: SR, 0.5 mg/mL; ATP, 100 μM; EGTA, 100 μM; $CaCl_2$, 61 μM (1 μM free Ca^{2+}) (●, ○), 88 μM (4 μM free Ca^{2+}) (■, □), or 98 μM (8 μM free Ca^{2+}) (▲, △). (Inset) Recalculated curves for control and phosphorylated SR using the normalization factors 2.32 for control SR and 1.73 for phosphorylated SR assayed at 1 μM free Ca^{2+} and 1.12 for control SR and 1.18 for phosphorylated SR assayed at 4 μM free Ca^{2+}.

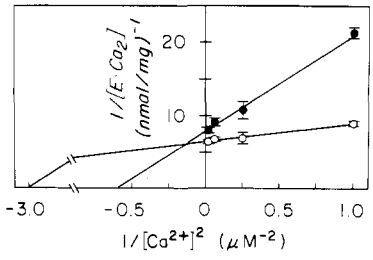

FIGURE 6: Double-reciprocal plot of $[E{\cdot}Ca_2]$ vs. $[Ca^{2+}]^2$. The levels of $E{\cdot}Ca_2$ were calculated as described under Methods. These data represent the mean ± SEM for seven determinations each for control (closed symbols) and phosphorylated (open symbols) SR.

(Figure 6). Also, the active-site density ($[E{\cdot}Ca_2]_{max}$) is higher in phosphorylated (0.16 nmol/mg) than control (0.13 nmol/mg) SR. These results indicate that phosphorylation regulates the calcium affinity and effective calcium binding sites of the calcium pump in cardiac SR. Our results of data analysis are in very good agreement with recent findings of Inesi et al. (1980) that, in $E + Ca \rightleftarrows E{\cdot}Ca \rightleftarrows E{\cdot}Ca_2$, the dissociation constant for the first step is 420 times as high as that for the second step with a Hill coefficient n = 1.82.

Effect of Phosphorylation by cAMP-Dependent Protein Kinase on Decomposition of E~P. Decomposition of E~P was measured from 0 to 73 ms under conditions which prevented further formation of E~P by the addition of excess EGTA. The decomposition of E~P follows first-order kinetics for at least the first 60% (Sumida et al., 1980). Phosphorylation by protein kinase resulted in stimulation of E~P decomposition with a twofold increase in the decomposition rate constant k_d. The time courses of decomposition of control and phosphorylated cardiac SR are shown in Figure 7. The rate constants of E~P decomposition are 4.8 ± 0.4 for control and 7.9 ± 0.7 for phosphorylated SR. The decomposition rate constant of untreated SR (same SR preparation, kept at 0 °C) was not significantly different from that of the control SR.

Discussion

In this study we examined the effect of phosphorylation of cardiac sarcoplasmic reticulum (SR) by cAMP-dependent protein kinase on the rates of phosphorylation and dephosphorylation of the enzyme intermediate (E~P) of the Ca^{2+}-ATPase. The rate of phosphorylation of the Ca^{2+}-AT-

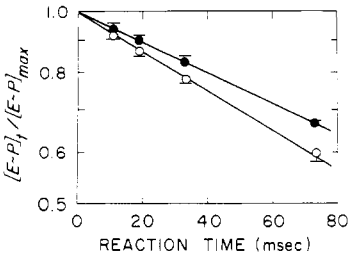

FIGURE 7: Effect of cAMP-dependent phosphorylation of cardiac SR on the time course of decomposition of the phosphorylated enzyme intermediate, E∼P, of the Ca^{2+}-ATPase. Cardiac SR preincubated in the presence (open symbols) and absence (closed symbols) of ATP was assayed for E∼P formation as described under Methods. Reaction conditions were 0.6 mg/mL SR, 100 μM ATP, 100 μM EGTA, and 100 μM $CaCl_2$ (10 μM free Ca^{2+}). After 116 ms, EGTA (2 mM final) was added, and the reaction was terminated after an additional variable incubation period (3.6–73 ms) by the addition of 9% perchloric acid and 6 mM phosphate. The data represent the mean ± SEM for three determinations.

Pase was examined by determining the levels of E∼P formed from enzyme preincubated with calcium at various concentrations. The phosphorylation (stable phosphoester) of cardiac SR by cAMP-dependent protein kinase resulted in stimulation of the initial rate as well as steady-state levels of E∼P. However, the half-time for approach of the maximal E∼P level was the same for control and phosphorylated cardiac SR, indicating a possible increase in the apparent active-site density of the Ca^{2+}-ATPase due to phosphorylation by protein kinase. These findings are different from those recently reported by Tada et al. (1979), who reported that phosphorylation of SR by protein kinase decreases the steady-state levels of E∼P especially when assayed at low Ca^{2+} concentrations (0.1–10 μM). The increase in E∼P levels we report here is not due to an increase in the apparent rate constant for E∼P formation, k (eq 2), but can be accounted for by an increase in the affinity of the enzyme for calcium and the increased active-site density observed under pre-steady-state conditions (decreased K_d and increased $[E{\cdot}Ca_2]_{max}$, Figure 6). Such an increase in calcium affinity gives a greater portion enzyme in the active $E{\cdot}Ca_2$ form, which reacts with ATP to form E∼P and results in an increase in E∼P levels. Our findings are in agreement with those of other investigators who, measuring anion-supported Ca^{2+} uptake under steady-state conditions, suggested that cAMP-dependent phosphorylation of SR may regulate the calcium affinity and transport of the ATPase (Tada et al., 1974; Hicks et al., 1979).

We also examined the effect of cAMP-dependent phosphorylation on E∼P decomposition. E∼P decomposition is thought to be one of the slow steps in the Ca^{2+}-ATPase reaction sequence (Tada et al., 1978), and it may regulate the overall enzymatic activity. We have recently shown that E∼P decomposition of the cardiac Ca^{2+}-ATPase is biphasic (Sumida et al., 1980). In the present study we examined the effect of phosphorylation by protein kinase on the initial phase (0–73 ms) of E∼P decomposition and found that the cAMP-dependent phosphorylation stimulates E∼P decomposition (k_d = 4.82 and 7.89 s^{-1} for control and phosphorylated SR, respectively). In addition to the present work, Tada et al. (1979) showed that the stimulation also occurred in the second phase of E∼P decomposition when measurement of the E∼P level started at 500 ms after its formation had been terminated (k_d = 1.05 and 1.78 s^{-1} for control and phosphorylated SR). In view of the time course of the cardiac contraction–relaxation cycle, the data on initial E∼P decomposition, in 0–73 ms time range, may be a better indication of the possible stimulating role of the protein kinase mediated phosphorylation of cardiac SR in vivo.

The present findings suggest that the Ca^{2+}-ATPase enzyme itself is directly affected by protein kinase mediated phosphorylation of the SR membranes. Enhancement of any of the steps in eq 1 could account for the observed stimulation of the calcium pump in vitro (La Raia & Morkin, 1974; Kirchberger et al., 1974). We have shown here that both the affinity and the active-site density of the enzyme for calcium and the rate of E∼P decomposition are regulated by protein kinase. Stimulation of these steps by protein kinase may account for the increase in V_{max} of the Ca^{2+}-ATPase and the rate of calcium transport when the cardiac SR is phosphorylated by protein kinase.

The alterations in the Ca^{2+}-ATPase reaction steps which we report here represent neither nonspecific changes due to exposure of SR to different ionic conditions during preincubation with protein kinase nor changes occurring during washing of the SR. Control SR was preincubated under the same conditions as phosphorylated SR but in the absence of ATP to prevent protein kinase mediated phosphorylation. Previously, EDTA was used in the preincubation of control SR to minimize the protein kinase activity (Tada et al., 1979). However, we observed that the presence of EDTA in the preincubation medium caused an inhibition of the E∼P levels which could lead to the false assumption that phosphorylation (carried out in the absence of EDTA) causes an increase in E∼P levels. Therefore, EDTA was not used in the present study. It has also been recently reported that cAMP alone increases the calcium permeability of the SR (Weller & Laing, 1979). In this study cAMP was present in the preincubation reaction for both control and phosphorylated SR. Therefore, the changes observed cannot be attributed to cAMP alone. Additionally, the phosphorylated and nonphosphorylated states of cardiac SR by protein kinase were carefully controlled by monitoring the amounts of phosphoester formation in the presence of [γ-^{32}P]ATP. Therefore, we attribute the observed alterations in SR activity to changes associated with cAMP-dependent protein kinase catalyzed phosphorylation of SR. The mechanism of these alterations is not known.

These findings indicate that phosphorylation by cAMP-dependent protein kinase can regulate the rate of the Ca^{2+}-ATPase in cardiac SR. Such regulation is found to be more effective at low Ca^{2+} concentrations where E∼P formation is slower than at higher Ca^{2+} concentrations. It appears that cAMP-dependent phosphorylation results in an apparent increase in the rate of turnover of the Ca^{2+}-ATPase and may account for the positive inotropic effect of catecholamines. However, proof that phosphorylation of cardiac SR regulates calcium transport must await isolation of the protein substrate and determination of the effect of its phosphorylation on the Ca^{2+}-ATPase activity in a reconstituted calcium pump. Phosphorylation of the SR must be also demonstrated in vivo in response to elevating catecholamines, and a correlation between the degree of phosphorylation and contractility must be established.

Added in Proof

After submission of this manuscript, a paper was published by Tada and his colleagues (Tada et al., 1980) in which it was shown that phosphorylation of cardiac SR by cAMP-dependent protein kinase enhances the rate of E∼P formation of "calcium free" SR in the transient state. These data in the millisecond time range are different from those of their previous publication (Tada et al., 1979) in which no change in rate and a drop in levels of E∼P were observed when the first

time point reported was at 500 ms.

Acknowledgments

We thank Dr. Steven Robertson for the use of his computer program for the calculations of free calcium concentrations and Dr. Earl T. Wallick for valuable discussion. We also thank L. I. Tsai and M. G. Pease for technical assistance.

References

Froehlich, J. P., & Taylor, E. W. (1975) *J. Biol. Chem. 250*, 2013–2021.
Hicks, M. J., Shigekawa, M., & Katz, A. M. (1979) *Circ. Res. 44*, 384–391.
Inesi, E., Kurzmack, M., Coan, C., & Lewis, D. E. (1980) *J. Biol. Chem. 255*, 3025–3031.
Kirchberger, M. A., & Tada, M. (1976) *J. Biol. Chem. 251*, 725–729.
Kirchberger, M. A., Tada, M., & Katz, A. M. (1974) *J. Biol. Chem. 249*, 6166–6173.
Kirchberger, M. A., Tada, M., & Katz, A. M. (1975) *Recent Adv. Stud. Card. Struct. Metab. 5*, 103–115.
Kranias, E. G., Mandel, F., & Schwartz, A. (1980) *Biochem. Biophys. Res. Commun. 92*, 1370–1376.
Laemli, U. K. (1970) *Nature (London) 227*, 680–685.
LaRaia, P. J., & Morkin, E. (1974) *Circ. Res. 35*, 298–305.
Maizel, J. V., Jr. (1969) in *Fundamental Techniques in Virology* (Habel, K., & Salzman, J. P., Eds.) pp 334–362, Academic Press, New York.
Schwartz, A., Entman, M. L., Kaniike, K., Lane, L. K., Van Winkle, W. B., & Bornet, E. P. (1976) *Biochim. Biophys. Acta 426*, 57–72.
Sillen, L. G., & Martell, A. E. (1964) in *Stability Constants of Metal-Ion Complexes*, 2nd ed., Ser. Publ. No. 17, The Chemical Society, Burlington House, London, WIV OBN.
Sumida, M., Wang, T., Mandel, F., Froehlich, J. P., & Schwartz, A. (1978) *J. Biol. Chem. 253*, 8772–8777.
Sumida, M., Wang, T., Schwartz, A., Younkin, C., & Froehlich, J. P. (1980) *J. Biol. Chem. 255*, 1497–1503.
Tada, M., Kirchberger, M. A., Repke, D. I., & Katz, A. M. (1974) *J. Biol. Chem. 249*, 6174–6180.
Tada, M., Kirchberger, M. A., & Katz, A. M. (1975) *J. Biol. Chem. 250*, 2640–2647.
Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol. Rev. 58*, 1–79.
Tada, M., Ohmori, F., Yamada, M., & Abe, H. (1979) *J. Biol. Chem. 254*, 319–326.
Tada, M., Yamada, M., Ohmori, F., Kuzuya, T., Inui, M., & Abe, H. (1980) *J. Biol. Chem. 255*, 1985–1992.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weller, M., & Laing, W. (1979) *Biochim. Biophys. Acta 551*, 406–419.
Will, H., Blanck, J., Smettan, G., & Wollenberger, A. (1976) *Biochim. Biophys. Acta 449*, 225–303.
Wray, H. L., & Gray, R. R. (1977) *Biochim. Biophys. Acta 461*, 441–459.

Quantitative Analyses of Calcium-Induced Spectral Changes in Extrinsic Cotton Effects of Cobalt-Substituted Concanavalin A†

Alan D. Cardin,‡ W. David Behnke,* and Frederic Mandel

ABSTRACT: The visible cobalt circular dichroism (CD) of cobalt-substituted concanavalin A (Con A) is highly sensitive to Ca^{2+}-induced conformational changes that occur in the metal binding region. The observed ellipticity is separately resolved into discrete conformational spectra with separate extrinsic bands. The conformational forms of the metal region are further delineated on the basis of their differential spectral response to the competitive removal of metals by ethylenediaminetetraacetic acid (EDTA). The spectral forms sensitive to the effects of EDTA, cobalt–Con A (CPS, $\epsilon_{CPS}^{470} = 215$ M^{-1}) and calcium–cobalt–Con A (CaCPS, $\epsilon_{CaCPS}^{470} = 141$ M^{-1}), exhibit both unique extinctions and band shapes in the 400–600-nm region, as does the fully metalized EDTA-resistant species CaCPR ($\epsilon_{CaCPR}^{470} = 54$ M^{-1}). Equations describing the time dependence of the observed ellipticity have been derived in terms of the kinetic scheme, CPS + Ca ⇋ CaCPS ⇋ CaCPR, in which the second equilibrium is slow compared to the first. The above assignments allow a more complete quantitative description of the changes in CD amplitudes and band shapes due to Ca^{2+} binding and thus facilitate the understanding of Ca^{2+} interactions. Calcium binds to 0.93 Ca^{2+} site/25 500 M_r in CaCPS with a K_d for Ca^{2+} = 2.1×10^{-3} M at pH 5.3 and 25 °C. The interaction of Ca^{2+} with CPS to form CaCPS occurs at two equivalent and noninteracting S_2 sites each present on separate subunits of the Con A dimer. Furthermore, the rate constant describing the rate of formation of CaCPR was determined.

Concanavalin A (Con A), a metallolectin isolated from the Jack bean *Canavalia ensiformis* (Sumner & Howell, 1936), has a substrate specificity for carbohydrate structures bearing the D-arabinopyranoside configuration (Goldstein et al., 1965, 1973). This protein has been the subject of numerous biochemical studies owing primarily to its ability to bind certain cell-surface carbohydrate acceptors although its true biological function is not known (Bittiger & Schnebli, 1976; Lis & Sharon, 1973). Among the many interesting effects induced by Con A binding is its ability to agglutinate selectively different cell types (Inbar & Sachs, 1969) and to elicit a mitogenic response in lymphocytes (Beckert & Sharkey, 1970; Powell & Leon, 1970; Wecksler et al., 1968).

The metal binding region of Con A has been a subject of particular interest, as it has a structural relation to the region where saccharides are bound. Each protomeric unit of Con

† From the Department of Biological Chemistry (A.D.C. and W.D.B.) and the Department of Pharmacology and Cell Biophysics (F.M.), University of Cincinnati College of Medicine, Cincinnati, Ohio 45267. *Received March 3, 1980.* Supported in part by the National Institutes of Health (HL 23741).

‡ In partial fulfillment of the degree Doctor of Philosophy.

0006-2960/80/0419-5439$01.00/0