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The Effect of cAMP-Dependent Protein Kinase Phosphorylation on the External Ca^{2+} Binding Sites of Cardiac Sarcoplasmic Reticulum

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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 is associated with an increase in both the initial rate of Ca^{2+} uptake and the Ca^{2+}-ATPase activity which is partially due to an increase in the affinity of the Ca^{2+}-Mg^{2+}-ATPase (E) of sarcoplasmic reticulum for calcium. In this study, the effect of cAMP-dependent protein kinase phosphorylation on the binding of calcium to the SR and on the dissociation of calcium from the SR was examined. The rate of dissociation of the $E \cdot Ca_2$ was measured directly and was not found to be significantly altered by cAMP-dependent protein kinase phosphorylation. Since the affinity of the enzyme for Ca^{2+} is equal to the ratio of the on and off rates of calcium, these results demonstrate that the observed change in affinity must be due to an increase in the rate of calcium binding to the Ca^{2+}-Mg^{2+}-ATPase of SR. In addition, an increase in the degree of positive cooperativity between the two calcium binding sites was associated with protein kinase phosphorylation.

Key Words: Ca^{2+} binding; Ca^{2+}-Mg^{2+}-ATPase; sarcoplasmic reticulum; cAMP; protein kinase; cooperativity; cardiac muscle; membrane protein; kinetics.

Introduction

Calcium and cyclic AMP (cAMP) are well-known modulators of myocardial contractility (Sandow, 1965; Robinson *et al.*, 1967; Ebashi and Endo, 1968; Katz, 1970; Rasmussen, 1970). Regulation by these two "second messengers" is believed to be achieved at least in part by augmenting calcium transport into the SR (LaRaia and Morkin, 1974; Kirchberger *et al.*, 1974; Schwartz *et al.*,

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1976; Will *et al.*, 1976). Calcium transport into the SR is mediated by an ATP-dependent calcium pump, the Ca^{2+}-Mg^{2+}-ATPase (Tada *et al.*, 1978). The characteristics of this enzyme (E) can be represented by the following reaction sequence (Froehlich and Taylor, 1975; Sumida *et al.*, 1978):

$$E' \underset{(1)}{\overset{+2Ca^{2+}}{\longleftrightarrow}} E \cdot Ca_2 \underset{(2)}{\overset{ATP}{\longleftrightarrow}} E \cdot Ca_2 \cdot ATP \underset{(3)}{\longleftrightarrow} ADP + Ca_2 \cdot E{\sim}P \underset{(4)}{\longleftrightarrow}$$

$$Ca_2E\text{-}P \underset{(5)}{\overset{-2Ca^{2+}}{\longleftrightarrow}} E' + P_i \quad (1)$$

A "regulator" for the Ca^{2+} pump in cardiac SR is a 22,000 M_r phosphoprotein, referred to as phospholamban, which has recently been shown to have a momomeric M_r of 8000–11,000 daltons (Le Peuch *et al.*, 1979; Jones *et al.*, 1981). Phospholamban is phosphorylated both by cAMP-dependent and Ca^{2+}-calmodulin-dependent protein kinases (LaRaia and Morkin, 1974; Kirchberger *et al.*, 1974; Schwartz *et al.*, 1976; Le Peuch *et al.*, 1979; Kranias *et al.*, 1980a). Cyclic AMP-dependent phosphorylation has been studied to a greater extent than Ca^{2+}-calmodulin-dependent phosphorylation, and it appears to be associated with an increase in Ca^{2+} transport and Ca^{2+}-ATPase activity by the SR (LaRaia and Morkin, 1974; Tada *et al.*, 1974; Schwartz *et al.*, 1976; Wray and Gray, 1977; Wollenberger and Will, 1978; Hicks *et al.*, 1979; Le Peuch *et al.*, 1979; Kranias *et al.*, 1980a). Recently, the effects of cAMP-dependent phosphorylation on the transient-state Ca^{2+}-ATPase activity have been reported. In particular, phosphorylation of phospholamban results in an increase in the apparent initial rates of E~P decomposition, and Ca^{2+} transported (Tada *et al.*, 1979; Tada *et al.*, 1980; Kranias *et al.*, 1980b). This stimulation is associated with a decrease in the dissociation constant for Ca^{2+} binding ($K_d = k_{off}/k_{on}$) (Kranias *et al.*, 1980b). In the present communication, we report that the decrease in K_d is probably due to an increase in the k_{on} since k_{off} was measured directly and no significant alteration of this rate constant due to cAMP-dependent phosphorylation was found.

Experimental Procedures

Materials

All biochemical reagents including beef heart cyclic AMP-dependent protein kinase were purchased from Sigma Chemical Co. The chemicals used were of chemically pure grade. Disodium ATP was purchased from Boehringer Mannheim and the ammonium salt of [γ-^{32}P]-ATP (10–40 Ci/mmol) was purchased from ICN.

Methods

Calcium Buffers. 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 using the association constants of Sillen and 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 100 μM ATP.

Preparation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum (SR) from dog cardiac muscle was prepared by the procedure of Harigaya and Schwartz (1969) with some modifications (Mandel *et al.*, 1982). The final yield was approximately 1 mg of SR protein per gram of wet tissue. The purity and homogeneity of the preparations were checked by electron microscopy after negative staining, by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and by various enzyme marker activities as previously described (Kranias *et al.*, 1980b).

Cyclic AMP-Dependent Protein Kinase Phosphorylation of Cardiac Sarcoplasmic Reticulum. Phosphorylation was carried out at 30°C in 50 mM phosphate buffer (pH 6.8) containing 10 mM $MgCl_2$, 1 mg/ml sarcoplasmic reticulum vesicles, and 500 μM ATP. The final concentrations of cyclic AMP (cAMP) and cAMP-dependent protein kinase were 1.0 μM and 50 μg or 100 picomolar units, respectively, per milliliter of reaction mixture. Phosphorylation of cardiac SR by cAMP-dependent protein kinase was not Ca^{2+}-activated in the absence of added calmodulin, indicating that the phosphorylation observed was due only to cAMP-dependent protein kinase (Kranias *et al.*, 1980a). Control, nonphosphorylated vesicles were treated under identical conditions without ATP. After 5 min of incubation, the mixtures were centrifuged at 105,000 $\times$ *g* for 30 min, washed twice, and the pellet was homogenized gently in ice-cold 20 mM Tris-maleate buffer (pH 6.8) containing 100 mM KCl. The recovery of phosphorylated and control SR protein after this procedure was about 85%. As we have previously shown (Kranias *et al.*, 1980b), the properties of control vesicles were not significantly different from those of untreated vesicles. To monitor the degree of SR phosphorylation, SR was incubated in the presence of [γ-^{32}P]-ATP (20 μCi/μmol) and treated as described above. The phosphoester bonds due to the protein kinase-mediated phosphorylation of SR were determined as previously described (Kranias *et al.*, 1980b) and they were found to be stable during centrifugation and washing of the phosphorylated SR. Control and phosphorylated SR were processed simultaneously and were assayed within 30 min of each other.

General Procedure for Transient-State Kinetic Studies. Rapid mixing experiments were performed using a previously described chemical quench

flow apparatus (Froehlich *et al.*, 1976; Sumida *et al.*, 1978). The device is equiped with five syringes and four mixers, which allows up to three substrates to be consecutively combined with the enzyme before the reaction is quenched. The standard vehicle solution for the SR and the substrates contained 0.1 M KCl, 3 mM $MgCl_2$, 5 mM NaN_3, and 20 mM Tris-maleate (pH 6.8). For these studies, two sets of experiments were performed. In the first set of experiments, the formation of E~P was determined as a function of time. Sarcoplasmic reticulum (~1.2 mg/ml concentration) was preincubated with a Ca^{2+}-EGTA buffer calculated (as described above) to contain various concentrations of free calcium. After 10–15 min of preincubation, this calcium-bound SR was loaded into the first syringe and was mixed in a 1:1 ratio with

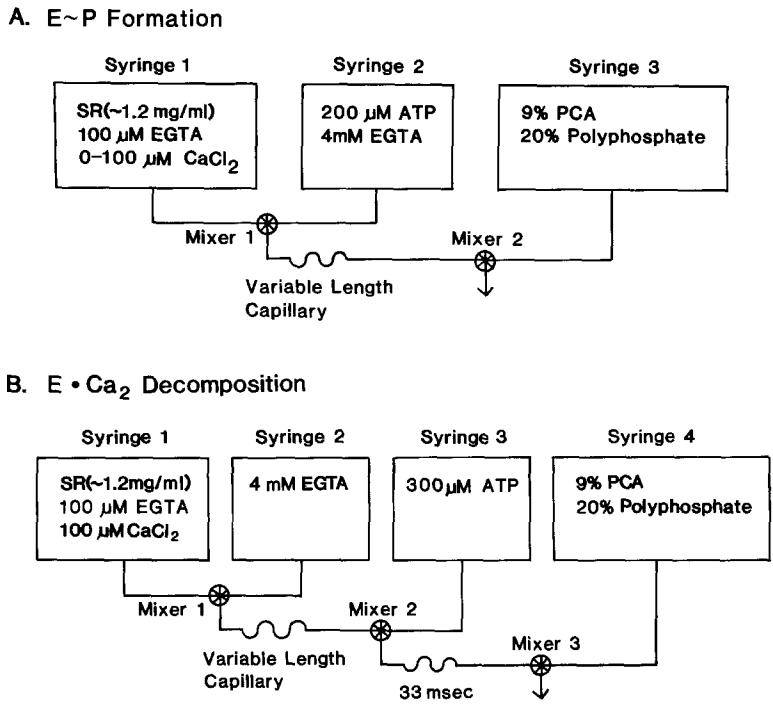

Fig. 1. (A) Schematic diagram of the quench-flow apparatus and the additions used for the measurement of the calcium affinity. The calcium affinity was determined by measuring the initial rates of E~P formation from SR preincubated at various free calcium concentrations. (B) Schematic diagram of the quench-flow apparatus and the solutions used to determine the rates of $E \cdot Ca_2$ decomposition. The vehicle solution used for all experiments contained 0.1 M KCl, 3 mM $MgCl_2$, 5 mM NaN_3, and 20 mM Tris-maleate, pH 6.8. The experiments shown in the other figures in this paper were performed under the same conditions unless otherwise stated. The free calcium concentration was adjusted by a calcium-EGTA buffer system. Inner diameter and lengths of capillaries between mixers are variable.

the solution in the second syringe containing 200 μM [γ-^{32}P] ATP (0.1–0.2 μCi/nmol ATP), and 4 mM EGTA. After a variable time interval, the reaction was quenched with a quenching solution consisting of 9% perchloric acid and 20% polyphosphate. A schematic diagram of this set of experiments which was used to measure the calcium dependence of the E~P formation rates is shown in Fig. 1A.

In a second set of experiments, the $E \cdot Ca_2$ formed from the preincubation of SR with 10 μM free calcium is loaded into the first syringe and is mixed with 4 mM EGTA (2 mM final) in order to chelate any free Ca^{2+} present and thereby to prevent any new $E \cdot Ca_2$ formation. The $E \cdot Ca_2$ complex initially present is allowed to decompose in the presence of EGTA for a variable (0–120 msec) time period before it is mixed in the second mixer with 300 μM ATP (100 μM ATP final). Upon the addition of ATP, the undissociated $E \cdot Ca_2$ reacts with the ATP to form E~P. The reaction is allowed to proceed for a time interval chosen to yield adequate quantities of E~P. We found that our results were independent of the time interval chosen between 20 and 50 msec. For convenience, a time of 33 msec was chosen. We shall use the term $E \cdot Ca_2$ decomposition to describe these experiments which are the results of both the dissociation of Ca^{2+} from the $E \cdot Ca_2$ complex as well as the concomitant conformational changes of the enzyme. A schematic diagram of the $E \cdot Ca_2$ decomposition experiments is shown in Fig. 1B.

For the isolation of phosphorylated enzyme (E~P), a constant volume (1.8 ml) of the assay solution was pipetted, stored in ice with 0.5 ml of carrier protein (1 mg/ml of skeletal SR), and centrifuged at 3000 rpm (Beckman RJ-6) for 5 min. The supernatant was discarded and the pellet was washed three times with 3 ml of washing solution containing 3% perchloric acid and 5% polyphosphate. The final pellet was dissolved in 1 ml of 10 mM NaOH and mixed with 9 ml of H_2O. The Cerenkov radiation from the ^{32}P was then counted in a Packard scintillation counter. The zero time blank was prepared by mixing the enzyme with perchloric acid prior to the addition of ATP and was subtracted from each sample.

Results

Effect of Phosphorylation by cAMP-Dependent Protein Kinase on the Calcium Dependence of the Initial Rates of E~P Formation. In our previous study (Kranias *et al.*, 1980b), we demonstrated that the initial rates of E~P formation were stimulated by cAMP-dependent protein kinase phosphorylation. SR was preincubated with various concentrations of free calcium (maintained by EGTA-Ca^{2+} buffers as described in Methods) for approximately 10–15 min at which time the binding of Ca^{2+} to the Ca^{2+}-

Mg^{2+}-ATPase is at equilibrium. This calcium-bound SR was then mixed in the quench-flow apparatus with ATP and an excess of EGTA. The formation of E~P was allowed to occur for up to 8.5 msec at which time the reaction was quenched with acid. Thus the following reaction was studied:

$$E' + 2Ca \underset{\text{equilibrium}}{\overset{\text{At}}{\longleftrightarrow}} E \cdot Ca_2 \overset{+ATP}{\longleftrightarrow} E\sim P \longleftrightarrow E + P_i \qquad (2)$$

Since the excess EGTA prevents the formation of any new $E \cdot Ca_2$ and since the decomposition rates of $E \cdot Ca_2$ (5–20 sec^{-1}) and E~P (5–15 sec^{-1}) are slow compared to the E~P formation rate (150–200 sec^{-1}) at short time intervals (0–10 msec), the reaction being studied can be expressed as

$$E \cdot Ca_2 \overset{k[ATP]}{\longleftrightarrow} E\sim P \qquad (3)$$

Thus, the expression for [E~P] as a function of time is

$$[E\sim P] = A\,(1 - e^{-k't}) \qquad (4)$$

where $A = [E \cdot Ca_2]$ at time zero and $k' = k\,[ATP]$. The values of A and k' at any given calcium and ATP concentration may be determined by nonlinear regression. Once values for A and k' are obtained, it is possible to obtain the rate constant for ATP binding simply by dividing k' by the ATP concentration. In addition, if A is known as a function of the free calcium concentration, it may be used to determine the affinity of the enzyme for calcium.

In our previous study (Kranias *et al.*, 1980b), we demonstrated that cAMP-dependent phosphorylation of cardiac SR resulted in stimulation of the E~P levels in the initial phase (4.3–8.5 msec) of the reaction at various free calcium concentrations. The data were analyzed with a nonlinear regression program to obtain values for k' and A. The apparent rate constant k' was found to be approximately 270 sec^{-1} for both control and phosphorylated SR, indicating that phosphorylation of SR by protein kinase does not significantly alter this rate constant. However, the values of A at any given $[Ca^{2+}]$ are significantly different for phosphorylated and control SR, indicating that phosphorylation does alter the affinity of the enzyme for calcium. Double reciprocal plots of the values of $1/A$ from our previous data versus $1/[Ca^{2+}]$ and $1/[Ca^{2+}]^2$ were of approximately equal linearity, and we were unable to discriminate between a noncooperative model and an infinitely cooperative model of calcium binding at the time. Thus, in the present study, we extended our studies of the initial rates of E~P formation to a lower free calcium concentration (0.63 μM) in order to better discriminate between the two models. The initial rates of E~P formation (0–8.5 msec) obtained at several representative calcium concentrations are shown in Fig. 2.

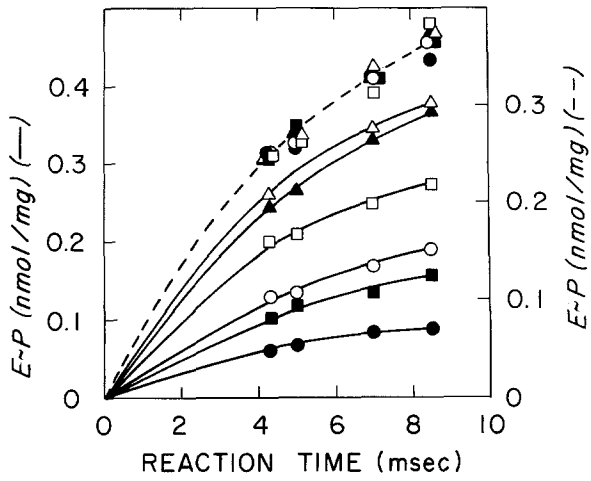

Fig. 2. Effect of cAMP-dependent phosphorylation of cardiac SR on the velocity of E~P formation. Phosphorylated (open symbols) and control (closed symbols) cardiac SR was assayed for E~P formation as described under Methods and as shown in Fig. 1A. Reaction conditions were: SR, 0.5 mg/ml; ATP, 100 μM; EGTA, 100 μM; $CaCl_2$, 50 μM (0.63 μM free Ca^{2+}) (●, ○), 61 μM (1 μM free Ca^{2+}) (■, □) and 100 μM (10 μM free Ca^{2+}) (▲, △). The dashed line represents renormalization of the data for phosphorylated (open symbols) and control (closed symbols) cardiac SR with respect to the 10 μM free Ca^{2+} results (▲). The shape of all the curves remains the same irrespective of the calcium concentration.

The Calcium Dependence of E~P Formation at 4 msec. An alternate procedure for the determination of the effect of cAMP-dependent phosphorylation on the calcium affinity of the SR is to examine the calcium dependence profile of the amount of E~P formed at 4.3 msec. The same experimental protocol was used as described above except that the reaction was allowed to proceed for only 4.3 msec prior to the PCA quench. We felt that at 4.3 msec (the fastest time available to us) the amount of E~P and $E \cdot Ca_2$ decomposed would be minimized and the amount of E~P formed would be a good indicator of the amount of $E \cdot Ca_2$ present at the start of the reaction. Thus, a plot of E~P versus the free calcium concentration will closely resemble a typical Ca^{2+} binding curve.

In a previous paper (Kranias *et al.*, 1980b), we used this procedure to compare the Ca^{2+}-binding properties of control SR (C-SR) and of SR phosphorylated by cAMP-dependent protein kinase (P-SR). Representative binding curves of C-SR and P-SR are shown in the inset of Fig. 3. In addition to a shift in the $K_{0.5}$ values of the two curves, there is also a marked change in the shape of the curves. Replots of these two curves as Hill plots are shown in

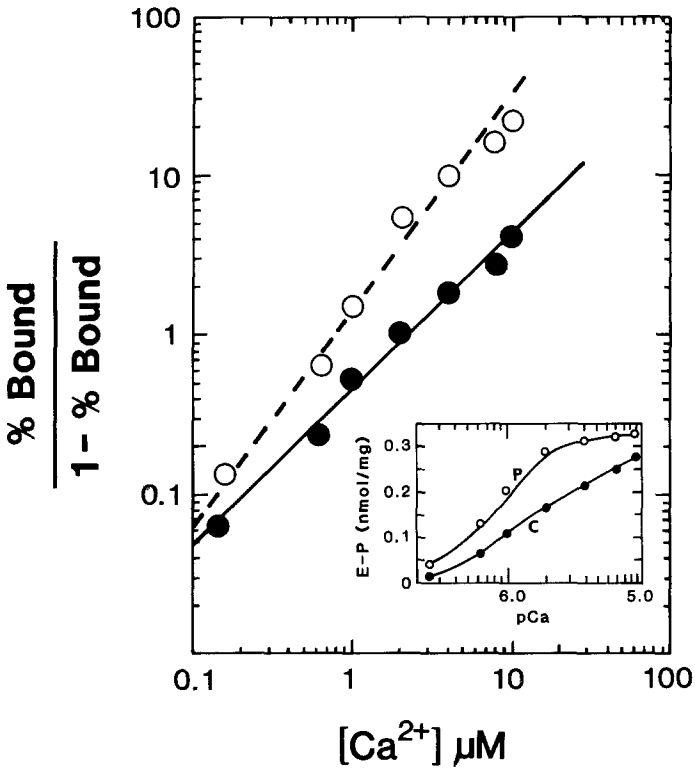

Fig. 3. Hill plots of calcium binding to the high-affinity calcium binding sites of phosphorylated (○) and control SR (●). The corresponding binding curves are shown in the inset. The binding was measured in the presence of 1–1.5 mg/ml SR and 100 μM ATP, as described in Methods and the caption of Fig. 1.

Fig. 3 and indicate that the apparent cooperativity between the two calcium binding sites has been altered. The nonphosphorylated binding curve had a Hill coefficient of 0.96 ± 0.19, while the phosphorylated SR yielded a Hill coefficient of 1.4 ± 0.2. Thus, our studies indicate that the degree of positive cooperativity between the calcium binding sites is enhanced as a result of cAMP-dependent protein kinase phosphorylation.

Analysis of the Binding Data. The general expression for ligand binding to multiple sites was first derived by Adair (1925) and is an N^{th} degree polynomial (where N equals the number of sites) in the ligand concentration. In addition, Hill (1910) demonstrated that binding curves can be approximately linearized by plotting the reciprocal of the concentration of

the bound species ($1/[E \cdot Ca_2]$) versus $1/[Ca^{2+}]^n$, where n is the well-known Hill coefficient. Furthermore, in the limits of infinite cooperativity, the Hill coefficient equals the number of sites ($n = 2$ in the present study), while in the absence of cooperativity $n = 1$. As discussed above, in our previous study we were unable to discriminate between these two models on the basis of reciprocal plots. This led us to extend our studies on the initial rates of E~P formation to a lower free calcium concentration (Fig. 2). Furthermore, the data in Fig. 2 (circles) along with the data from our previous studies (squares) were plotted vs. $1/[Ca^{2+}]$ (Fig. 4A) rather than $1/[Ca^{2+}]^2$ as they were in our earlier work (Kranias *et al.*, 1980b).

The value of the y intercept ($[Ca^{2+}] = 0$) denotes the reciprocal of the maximum number of calcium binding sites available (i.e., $1/[E \cdot Ca_2]_{max}$). Since the number of active Ca^{2+}-Mg^{2+}-ATPase molecules per milligram SR varies with the SR preparation used, this intercept will vary from experiment to experiment (just as if different amounts of SR of the same preparation were used). However, if the properties of the active Ca^{2+}-Mg^{2+}-ATPase molecules are invariant, then the x intercept, which is equal to the calcium affinity, should be independent of the preparation used. In both studies, the intercepts with the x axis (i.e., $-1/K_D$) of the C-SR occur at the same point (~ -0.33 μM^{-1}), and the x intercepts for the P-SR also occur at approximately the same point (~ -1.55 μM^{-1}). Thus, although two different SR preparations were used in these two experiments, the affinity of both the C-SR and P-SR for calcium is independent of the preparation used. Furthermore, although the y intercepts of the two different SR preparations differ markedly, the y intercepts of the control and the phosphorylated SR of each preparation are virtually identical. Thus, these data indicate that the number of calcium binding sites are not affected by protein kinase phosphorylation (Fig. 4A).

In order to facilitate the comprison of experiments performed on different days with different preparations, the results were normalized with respect to the values of the respective y intercepts. All the data from one experiment (circles) were multiplied by a factor of 2.8 (ratio of the y intercepts) in order to have identical y intercepts with the other experiment (squares). The normalized data (Fig. 4B) appear linear and result in K_D values for phosphorylated and control SR of 0.7 and 2.6 μM, respectively. Since our previous data (squares) also exhibited a high degree of linearity when plotted versus $1/[Ca^{2+}]^2$, our present results were also plotted versus $1/[Ca^{2+}]^2$ in order to determine if our data at low calcium concentrations enable us to distinguish between the two models. The normalized data in Fig. 4B was replotted versus $1/[Ca^{2+}]^2$ in order to determine if the linear relationship still holds (Fig. 4C). It is obvious from Fig. 4C that the data for C-SR at 0.63 μM free Ca^{2+} no longer falls on a straight line. Thus, with the additional data, it is

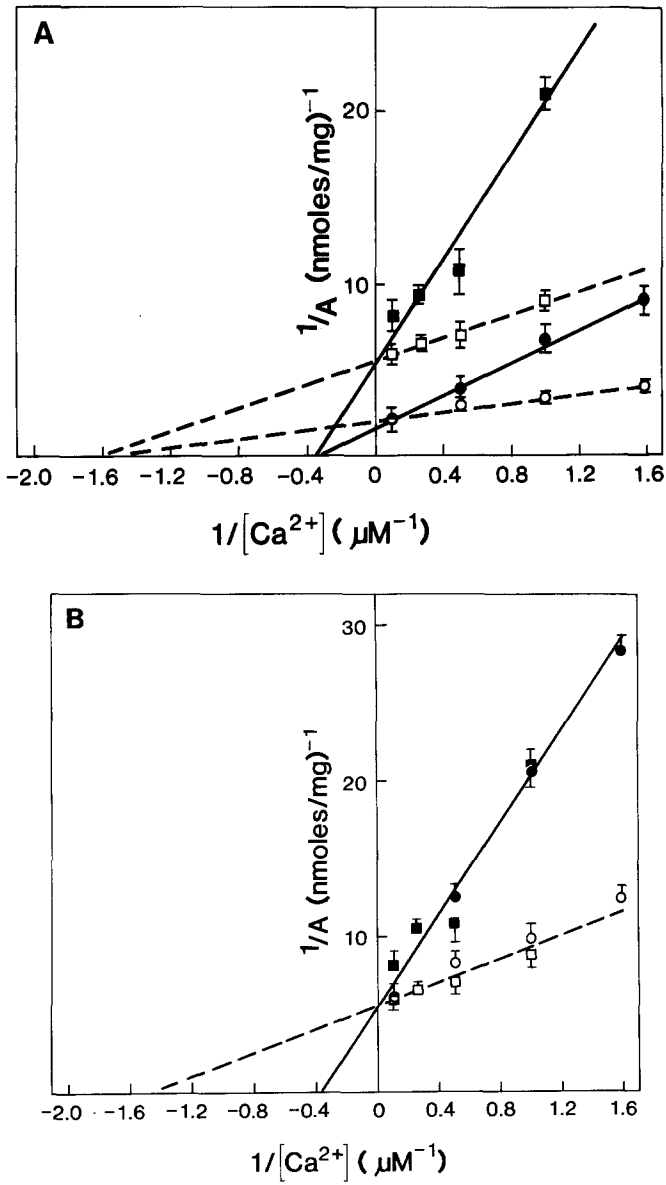

Fig. 4. (A) Double reciprocal plots of $[E \cdot Ca_2]$ at zero time (A) vs. [Ca] concentration for control (●, ■) and phosphorylated (○, □) SR. The circles are the data shown in Fig. 2 and the squares are data from our previous paper (Kranias *et al.*, 1980b). The values of A at various free calcium concentrations were calculated as discussed under Results and Discussion. These data represent the mean ± S.E.M. for seven determinations each. (B) Normalized double reciprocal plots of the data in Fig. 4A. The curves were normalized such that the y intercepts (the active-site densities) of the two experiments coincided. (C) The data in Fig. 4A replotted vs. $1/[Ca^{2+}]^2$. (D) The normalized data for the phosphorylated SR of Fig. 4B replotted vs. $1/[Ca^{2+}]^{1.4}$.

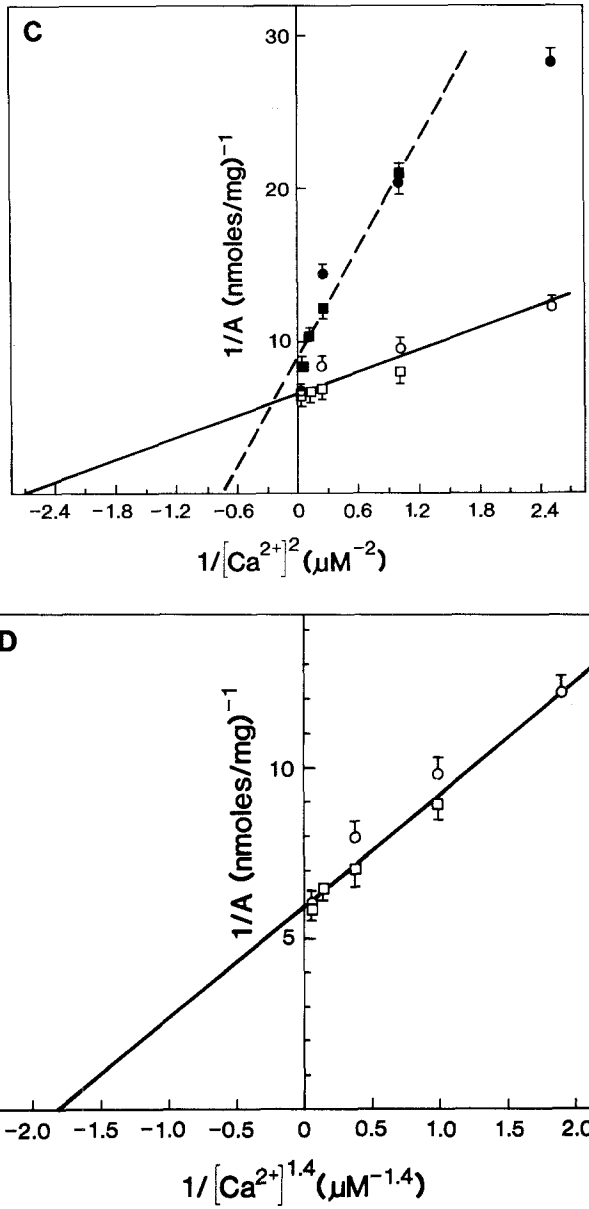

Fig. 4. Continued.

now possible to distinguish between the two models. Furthermore, when $1/A$ is plotted versus $1/[Ca^{2+}]^2$, the y intercepts no longer coincide for P-SR and C-SR.

For data with a Hill coefficient of 1.4, such as we have observed for P-SR, the highest degree of linearity would occur for a plot of $1/A$ vs. $1/[Ca^{2+}]^{1.4}$. Furthermore, the best value of the apparent K_D would be obtained from the x intercept of such a double reciprocal plot. In Fig. 4D, a normalized double reciprocal plot of $1/A$ versus $1/[Ca^{2+}]^{1.4}$ is shown for the P-SR data from which an apparent $K_D = 0.75 \pm 0.15$ μM was obtained. Thus, the apparent affinity of the Ca^{2+}-Mg^{2+}-ATPase for calcium is increased subsequent to cAMP-dependent protein kinase phosphorylation. Since the affinity is the quotient of the on and off rates of Ca^{2+}, the $E \cdot Ca_2$ decomposition was studied in order to determine whether the off rates of Ca^{2+} are affected by cAMP-dependent protein kinase phosphorylation.

Dissociation of Bound Ca^{2+} from SR. To measure the rates of $E \cdot Ca_2$ decomposition for C-SR and P-SR, we obtained the levels of E~P (max.) formed as a function of time subsequent to addition of 4 mM (2 mM final) EGTA as schematically shown in Fig. 1B. In these studies, incubation of the $E \cdot Ca_2$ complex (obtained by preincubating SR with 10 μM free Ca^{2+}) with 2 mM EGTA for various times (0–116 msec) results in a gradual decrease in the amount of Ca^{2+} bound to the SR due to the dissociation of Ca^{2+} from $E \cdot Ca_2$. Upon the addition of 300 μM (100 μM final) ATP, the externally bound Ca^{2+} is rapidly translocated into the interior of the vesicle concomitantly with the formation of E~P. Due to the presence of excess EGTA, the only source of calcium available for the formation of E~P is this "tightly bound" calcium. Consequently, the maximum level of E~P formed may be used as an appropriate measure of the initial amount of the SR:calcium complex. A semilogarithmic plot of E~P (max.) versus the incubation time of $E \cdot Ca_2$ with EGTA for C-SR and P-SR is shown in Fig. 5. We find that there is no significant difference in the initial rates (0–116 msec) of $E \cdot Ca_2$ decomposition between phosphorylated and nonphosphorylated cardiac SR. In order to ascertain that the SR used in the decomposition experiment was indeed stimulated by protein kinase phosphorylation, we also determined the initial rates of E~P formation for that SR preparation, as schematically shown in Fig. 1A. Our results are presented in the inset to Fig. 5 and show that at 2 μM free Ca^{2+}, the initial rate (0–19 msec) of E~P formation is significantly greater for the phosphorylated SR. Thus, using the same control and phosphorylated SR preparations, we see a stimulation of the initial rates of E~P formation without observing a concomitant change in the $E \cdot Ca_2$ decomposition rates. The results of the experiments shown in Fig. 5 and of three other $E \cdot Ca_2$ decomposition studies yielded a k_{off} for $E \cdot Ca_2$ decomposition of 8 ± 2 sec^{-1} for both C-SR and P-SR.

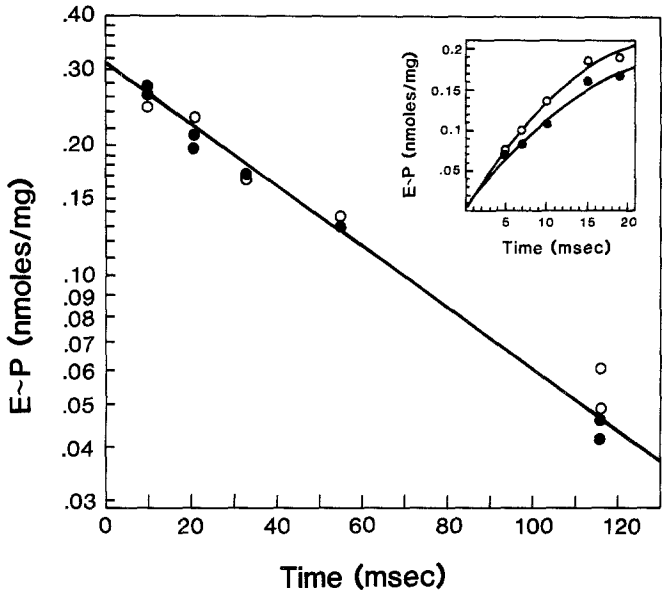

Fig. 5. Effect of cAMP-dependent phosphorylation of cardiac SR on the time course of the decomposition of $E \cdot Ca_2$. The $E \cdot Ca_2$ decomposition was assayed as discussed in Methods and as shown in Fig. 1B. Reaction conditions were 0.6 mg/ml SR, 100 μM ATP, 100 μM EGTA, and 100 μM $CaCl_2$ (10 μM free Ca^{2+}). This figure is one representative experiment for control (●) and phosphorylated (○) SR. The inset shows the E~P formation curves (0–19 msec) for the same control (●) and phosphorylated (○) SR preparation as the $E \cdot Ca_2$ decomposition results. The free calcium concentration was 2 μM (100 μM EGTA and 76.3 μM Ca^{2+}), and E~P formation was measured as described in Methods and as shown in Fig. 1A.

Discussion

Several studies (Sumida *et al.* 1978; Dupont and Leigh, 1978; Inesi *et al.*, 1980) have shown that the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+}-ATPase [step (1) in Eq. (1)] is composed of several steps including a slow conformational step. Inesi *et al.* (1980) have suggested the following reaction sequence for Ca^{2+} binding to rabbit skeletal SR:

$$E' \xleftrightarrow{+Ca^{2+}} E' \cdot Ca \longleftrightarrow E'' \cdot Ca \xleftrightarrow{+Ca^{2+}} E \cdot Ca_2 \qquad (5)$$

Using both equilibrium ^{45}Ca binding and the analysis of EPR spectra obtained from spin labeled SR, they (Inesi *et al.*, 1980) observed a high degree of cooperativity between the two Ca^{2+} binding sites resulting in a two orders of

magnitude increase in the affinity of the second site upon Ca^{2+} binding to the first site. In addition, Dupont and Leigh (1978) observed slow changes in the intrinsic fluorescence of SR due to the addition and removal of Ca^{2+}. Using rapid quench-flow techniques, we (Sumida *et al.*, 1978) examined the binding of Ca^{2+} to and the dissociation of Ca^{2+} from both cardiac and skeletal SR. Our results indicated a slow conformational change upon Ca^{2+} binding which was significantly slower for cardiac (12 sec^{-1}) than for skeletal (63 sec^{-1}) sarcoplasmic reticulum. This difference in rates implies a significant difference between the conformational changes that occur in cardiac and skeletal sarcoplasmic reticulum. In addition, we observed a difference in the $E \cdot Ca_2$ decomposition rates of cardiac and skeletal SR.

The present study indicates that cAMP-dependent phosphorylation of phospholamban has an effect on the binding of calcium to the high-affinity binding sites of the Ca^{2+}-Mg^{2+}-ATPase of cardiac SR. The dissociation constant of these sites for calcium is decreased upon phosphorylation of phospholamban from 2.6 ± 0.3 μM for C-SR to 0.75 ± 0.15 μM for P-SR. These results are in excellent agreement with those of Hicks *et al.* (1979) who observed a change in K_{Ca} from 2.38 ± 0.21 for C-SR to 1.07 ± 0.10 for phosphorylated SR.

The Hill coefficients of phosphorylated cardiac SR and of control (treated identically to the phosphorylated SR but in the absence of ATP) cardiac SR were approximately 1.4 and 1.0, respectively (Fig. 3). Similar quench-flow binding experiments with untreated cardiac SR and with rabbit skeletal SR yielded Hill coefficients of approximately 1.3 ± 0.4 and 2.0 ± 0.2, respectively (unpublished observations). Thus, protein kinase phosphorylation of cardiac SR results in increased cooperativity between the two Ca^{2+} binding sites of the Ca^{2+}-Mg^{2+}-ATPase and in "behavior" of the cardiac SR more like that of skeletal SR. This is qualitatively in agreement with the studies of Hicks *et al.* (1979) who also observed that upon phosphorylation the "behavior" of cardiac SR becomes more like that of fast skeletal sarcoplasmic reticulum. However, in contrast to our results, Hicks *et al.* (1979) found that the positive cooperativity of the calcium pump is decreased from 1.77 ± 0.15 to 1.24 ± 0.08. It is interesting to note that while our results and those of Inesi *et al.* (1980) yield a Hill coefficient of approximately 2.0 for skeletal SR, Hicks *et al.* observed a Hill coefficient of approximately unity for skeletal SR. This apparent contradiction in the observed cooperativity is addressed in a recent paper by Highsmith (1982) where it is shown that the degree of cooperativity of skeletal SR may depend on whether the SR has been preincubated in the presence of high or low concentrations of calcium prior to calcium binding. It is not presently known whether cardiac SR exhibits a similar behavior. Thus, the differences in Hill coefficients between our results and those of Hicks *et al.* may be due to different processing of the SR prior to

the experiment. In addition, it must be noted that while our studies and those of Inesi *et al.* were concerned with the binding of Ca^{2+} to the Ca^{2+}-Mg^{2+}-ATPase, the studies by Hicks *et al.* (1979) were done at steady state. Since steady-state uptake rates are composite functions of the entire Ca^{2+}-Mg^{2+}-ATPase reaction cycle and not necessarily just a function of Ca^{2+} binding of the high-affinity external binding sites, one would not necessarily expect the same functional dependence to hold in both cases. In fact, protein kinase phosphorylation has been shown to affect several steps in the SR reaction mechanism (Tada *et al.*, 1980; Kranias *et al.*, 1980b), and thus the net effect on steady-state parameters may be quite complex.

Since the affinity is the quotient of the "on rate" of Ca^{2+} divided by the "off rate," an increase in affinity can be due to either an increase in the "on rate," a decrease in the "off rate," or a combination of changes in both rates. In order to further elucidate the mechanism of action by which phosphorylation increases the Ca^{2+} sensitivity of SR, we directly measured the Ca^{2+} "off rate." Our studies (Fig. 5) indicate that protein kinase does not significantly alter the rate of dissociation of Ca_{2+} from the $E \cdot Ca_2$ complex. In addition, the $E \cdot Ca_2$ decomposition results shown in Fig. 5 are both linear, indicating that the change in cooperativity is not due to an interaction between the dissociation rates of the two calcium ions. Thus, the observed alterations in Ca^{2+} affinity are probably due to changes in the mechanism by which Ca^{2+} binds to the enzyme. An increase, upon phosphorylation, in the rate of the slow conformational change [second step in Eq. (5)] would result in an increased Ca^{2+} affinity such as we have observed. Stopped-flow experiments, such as those done by Guillain *et al.* (1981), directly measuring this conformational change for control and phosphorylated SR, would more clearly demonstrate the effect of phosphorylation.

Our present study provides further evidence that upon the formation of a phosphoester bond in phospholamban, a "message" is transmitted to the Ca^{2+}-Mg^{2+}-ATPase which results in an alteration of several of the intrinsic rate constants of the ATPase. Whether this message is transmitted by direct steric interaction with the Ca^{2+}-Mg^{2+}-ATPase, as suggested by Hicks *et al.* (1979), or indirectly through the lipid matrix is presently unknown.

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