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MECHANISM OF THE STIMULATION OF Ca^{2+}-DEPENDENT ATPase OF SKELETAL MUSCLE SARCOPLASMIC RETICULUM BY PROTEIN KINASE

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Sarcoplasmic reticulum isolated from moderately fast rabbit skeletal muscle contains intrinsic adenosine 3′,5′-monophosphate (cAMP)-independent protein kinase activity and a substrate of 100 000 M_r. Phosphorylation of skeletal sarcoplasmic reticulum by either endogenous membrane bound or exogenous cAMP-dependent protein kinase results in stimulation of the initial rates of Ca^{2+} transport and Ca^{2+}-ATPase activity. To determine the molecular mechanism by which protein kinase-dependent phosphorylation regulates the calcium pump in skeletal sarcoplasmic reticulum, we examined the effects of protein kinase on the individual steps of the Ca^{2+}-ATPase reaction sequence. Skeletal sarcoplasmic reticulum vesicles were preincubated with cAMP and cAMP-dependent protein kinase in the presence (phosphorylated sarcoplasmic reticulum) and absence (control sarcoplasmic reticulum) of adenosine 5′-triphosphate (ATP). Control and phosphorylated sarcoplasmic reticulum were subsequently assayed for formation (5–100 ms) and decomposition (0–73 ms) of the acid-stable phosphorylated enzyme (E ~ P) of Ca^{2+}-ATPase. Protein kinase mediated phosphorylation of skeletal sarcoplasmic reticulum resulted in pronounced stimulation of initial rates and levels of E ~ P in sarcoplasmic reticulum preincubated with either ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) prior to assay (Ca^{2+}-free sarcoplasmic reticulum), or with calcium/EGTA buffer (Ca^{2+}-bound sarcoplasmic reticulum). These effects were evident within a wide range of ionized Ca^{2+}. Phosphorylation of skeletal sarcoplasmic reticulum by protein kinase also increased the initial rate of E ~ P decomposition. These findings suggest that protein kinase-dependent phosphorylation of skeletal sarcoplasmic reticulum regulates several steps in the Ca^{2+}-ATPase reaction sequence which result in an overall stimulation of the active calcium transport observed at steady state.

Introduction

Phosphorylation of cardiac sarcoplasmic reticulum by cAMP-dependent protein kinases results in stimulation of Ca^{2+}-transport and Ca^{2+}-ATPase activity [1–6]. Phosphorylation occurs on a 22 kDa protein, referred to as phospholamban [7], which is thought to serve as modulator of the Ca^{2+}-ATPase in cardiac sarcoplasmic reticulum. Recently, the effect of protein kinase on the individual steps of the Ca^{2+}-ATPase reaction sequence has been reported. In particular, phosphorylation of phospholamban causes an increase in the initial rate and levels of E ~ P and an increase in the rate of E ~ P decomposition [8,9].

Although phosphorylation of cardiac sarcoplasmic reticulum and the resultant stimulation of

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

Ca^{2+}-transport have been well documented, similar possible reactions in fast skeletal muscle sarcoplasmic reticulum are not well known and in fact are the subject of considerable controversy [4,10–17]. There is accumulating evidence, however, that protein kinases are associated with fast skeletal sarcoplasmic reticulum [4,13–22], but the possible effects of these enzymes on the Ca^{2+} pump in a manner similar to cardiac sarcoplasmic reticulum have not been characterized. We have previously reported that skeletal sarcoplasmic reticulum contains an endogenous cAMP-independent protein kinase and a substrate for this activity of 100 000 M_r which do not appear to be due to contamination with sarcolemma [13]. Phosphorylation of the 100 kDa protein by either endogenous, membrane-bound, protein kinase or exogenous, soluble, cAMP-dependent protein kinase appears to be associated with stimulation of Ca^{2+}-transport and Ca^{2+}-ATPase activity [13]. It is possible, therefore, that the 100 kDa protein in fast skeletal sarcoplasmic reticulum acts as a regulator of the Ca^{2+} pump in a manner analogous to the 22 kDa protein, phospholamban, in cardiac sarcoplasmic reticulum.

To determine the molecular mechanism by which protein kinase-mediated phosphorylation of skeletal sarcoplasmic reticulum regulates the Ca^{2+}-ATPase we studied the effect of protein kinase on the individual steps of the Ca^{2+}-ATPase reaction. The objectives of the present study were 2-fold: (1) to elucidate the mechanism by which protein kinase may regulate skeletal sarcoplasmic reticulum and (2) to determine whether this mechanism is similar to the one previously reported for cardiac sacroplasmic reticulum [8,9].

It is generally accepted that the membrane-bound ($Ca^{2+} + Mg^{2+}$)-ATPase is responsible for the active transport of calcium into the sarcoplasmic reticulum lumen in cardiac and skeletal sarcoplasmic reticulum. The simplified reaction scheme of the Ca^{2+}-ATPase is shown in Eqn. 1 [23]:

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

The active transport of calcium is coupled to the formation of the phosphorylated intermediate of the Ca^{2+}-ATPase, E ~ P, even in the transient phase [24]. We studied the effect of protein kinase-mediated phosphorylation of rabbit skeletal sarcoplasmic reticulum on the Ca^{2+}-ATPase activity in the 0–100 ms time range. We report in the present paper that phosphorylation results in marked stimulation of the initial rates of both formation and decomposition of E ~ P. These findings indicate for the first time that protein kinase may regulate the elementary steps of the Ca^{2+}-ATPase activity in skeletal sarcoplasmic reticulum and the mechanism by which this regulation occurs is analogous to that for cardiac sarcoplasmic reticulum [8,9].

Methods

Materials. All biochemical reagents including bovine heart cAMP-dependent protein kinase were purchased from Sigma Chemical Co. The chemicals used were of 'chemical pure grade'. Disodium ATP was purchased from Boehringer Manheim and the ammonium salt of [γ-^{32}P]ATP (10–40 Ci/mmol), was purchased from ICN.

Miscellaneous methods. 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 [25]. 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 sacroplasmic reticulum vesicles. Sarcoplasmic reticulum from rabbit back skeletal muscle was prepared by the procedure of Harigaya and Schwartz [26] with some modifications as we previously described [24]. The final yield was 1.5 mg of sarcoplasmic reticulum 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 [13]. Cytochrome *c* oxidase activity per mg sarcoplasmic reticulum protein was less than 2% of that in the mitochondrial fraction (5000 $\times g$ pellet). Ouabain-

sensitive ($Na^+ + K^+$)-ATPase and [^{3}H]ouabain binding, enzymatic markers of sarcolemma, were less than 0.5% (0.005 mol active ($Na^+ + K^+$)-ATPase/mol active Ca^{2+}-ATPase) of the total ATPases of these membrane preparations. The sarcoplasmic reticulum ($Ca^{2+} + Mg^{2+}$)-ATPase (10 μg of protein/ml) was assayed in 50 mM Tris-maleate (pH 6.8) containing 5 mM $MgCl_2$, 100 mM KCl, 2 mM phospho*enol*pyruvate, 0.5 mM NADH, pyruvate kinase (7 units/ml), lactate dehydrogenase (10 units/ml), 5 mM NaN_3, 0.1 mM ouabain, 200 μM EGTA, 200 μM $CaCl_2$ (10 μM free Ca^{2+}) and 5 mM ATP [27]. The specific activity was about 200 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ 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 40–50 μmol $\cdot mg^{-1} \cdot h^{-1}$.

Phosphorylation of skeletal sarcoplasmic reticulum. Phosphorylation was carried out at 30°C in 50 mM phosphate buffer (pH 6.8) containing 10 mM $MgCl_2$, 0.5 mM EGTA, 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, respectively, per ml reaction mixture. Control vesicles were treated under identical conditions without ATP. After 5 min of incubation, the mixtures were centrifuged at 105 000 × *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 sarcoplasmic reticulum protein after this procedure was about 80%. To monitor the degree of sarcoplasmic reticulum phosphorylation, sarcoplasmic reticulum 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 sarcoplasmic reticulum were determined as previously described [13] and they were found to be stable during centrifugation and washing of the phosphorylated sarcoplasmic reticulum. The average value for 14 preparations was 150 pmol/mg sarcoplasmic reticulum protein. sarcoplasmic reticulum protein.

Transient state kinetic studies. Rapid mixing experiments were performed as previously described [8] using a chemical quench flow apparatus. The standard vehicle solution for the sarcoplasmic reticulum, 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. The acid-stable phosphorylated enzyme intermediate was quenched with perchloric acid and isolated for ^{32}P-counting [24].

Formation of phosphorylated intermediate of Ca^{2+}-ATPase in calcium-free and calcium-preloaded sarcoplasmic reticulum vesicles. For studies starting from calcium-free sarcoplasmic reticulum, the enzyme syringe contained rabbit skeletal sarcoplasmic reticulum vesicles (1–1.5 mg/ml) in the standard vehicle solution including 200 μM EGTA. The substrate syringe contained 200 μM ATP (including [γ-^{32}P]ATP) and various $CaCl_2$ concentrations in the standard vehicle solution, such that the mixture of enzyme and substrate yielded the desired [Ca^{2+}]. 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 5 to 100 ms. The zero-time blank prepared by mixing the enzyme with acid followed by substrate was subtracted from each sample. For determination of the initial rate of formation of the phosphorylated intermediate of Ca^{2+}-ATPase (E ~ P), E ~ P formation was allowed to proceed as described above for 5 ms, the first time point obtained in these studies.

For studies starting from calcium-preloaded sarcoplasmic reticulum vesicles, the enzyme syringe contained rabbit skeletal sarcoplasmic reticulum vesicles (1–1.5 mg/ml) in the standard vehicle solution including 100 μM EGTA and various $CaCl_2$ concentrations (free Ca^{2+} concentration of 0.1–100 μM). The substrate syringe contained 200 μM ATP (including [γ-^{32}P]ATP), 100 μM EGTA and various $CaCl_2$ concentrations (free Ca^{2+} concentration of 0.1–100 μM) 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 followed from 5 to 100 ms. For determination of the initial rate of formation of the phosphorylated intermediate of Ca^{2+}-ATPase

(E ~ P), an ATP (200 μM) solution containing EGTA (4 mM) was mixed with an equal volume of sarcoplasmic reticulum preincubated with Ca-EGTA buffer, which gave various concentrations of free Ca^{2+} (final 0.1–100 μM). Under such reaction conditions, EGTA chelates free Ca^{2+} to prevent the formation of new $E \cdot Ca_2$ during the course of E ~ P formation. The reaction was quenched with perchloric acid as described above.

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 skeletal sarcoplasmic reticulum 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 levels ($[E \sim P]_{max}$). Subsequent addition of perchloric acid to quench the E ~ P decomposition allowed measurement of the decreasing E ~ P level at various times, ranging from 4.3 to 73 ms. The rate of E ~ P decay was determined by plotting log $[E \sim P]_t/[E \sim P]_{max}$ versus time.

Results

Phosphorylation of skeletal sarcoplasmic reticulum by protein kinase

We have previously shown that rabbit skeletal sarcoplasmic reticulum, prepared as described in Methods, contains an endogenous cAMP-independent protein kinase and a substrate for this activity of 100000 M_r [13]. The 100 kDa protein was also phosphorylated by exogenous cAMP-dependent protein kinase, and this phosphorylation was abolished in the presence of the heat-stable protein kinase inhibitor isolated from rabbit skeletal muscle. The product of phosphorylation by endogenous or exogenous 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 sarcoplasmic reticulum vesicles were hydrolyzed by hot 0.5 N NaOH, but they were resistant to cleavage by 0.8 M hydroxylamine, indicating the presence of phosphoester bonds. Phosphorylation of skeletal sarcoplasmic reticulum by endogenous protein kinase activity was not Ca^{2+} activated. Furthermore, inclusion of calmodulin (10^{-8} to 10^{-6} M) in the presence of [Ca^{2+}] (10 μM) did not stimulate phosphorylation of the 100 kDa protein indicating that there was no Ca^{2+}-calmodulin-dependent protein kinase phosphorylation observed under these experimental conditions. Phosphorylation of the 100 kDa phosphoprotein by either endogenous or exogenous protein kinase appeared to be associated with stimulation of Ca^{2+} transport and Ca^{2+}-ATPase activity maintaining the stoichiometric coupling ratio of Ca^{2+}:ATP. Stimulation of the Ca^{2+} pump did not require either protein kinase or cAMP to be present at the time that measurements were made [13].

Transient-state Ca^{2+}-ATPase activity of sarcoplasmic reticulum phosphorylated by protein kinase

Skeletal sarcoplasmic reticulum was preincubated under each of the conditions used for phosphorylation as described under Methods. The reactants (ions, cAMP, protein kinase), temperature and time of incubation of the phosphorylation assay and subsequent washings of the sarcoplasmic reticulum had no effect on the rates of formation and decomposition of the phosphorylated intermediate of Ca^{2+}-ATPase (E ~ P). The amount of E ~ P (nmol/mg sarcoplasmic reticulum) formed as a function of time varied from preparation to preparation. However, the rate and levels of E ~ P formed and the rate of E ~ P decomposition for control sarcoplasmic reticulum were not significantly different from those of the same sarcoplasmic reticulum preparation which was not subject to preincubation. Thus, for each experiment performed on the effect of phosphorylation on the Ca^{2+}-ATPase parameters, the same sarcoplasmic reticulum preparation was used and

control and phosphorylated sarcoplasmic reticulum vesicles were processed simultaneously and assayed within 30 min of each other. The phosphoester bonds formed during preincubation of the skeletal sarcoplasmic reticulum with protein kinase were stable through washing and subsequent kinetic experiments. The amounts of ATP and P_i remaining with the phosphorylated sarcoplasmic reticulum after washing were less than 0.5% of the [ATP] (100 μM) added to the reaction medium for E ~ P formation. The effect of phosphorylation by protein kinase on Ca^{2+}-ATPase activity was also assayed under steady-state conditions for each sarcoplasmic reticulum preparation. Although steady-state studies were done under different conditions (see Methods) than transient state studies, the stimulatory effect of phosphorylation observed under steady-state conditions would reflect the effect on one or more of the individual steps of the Ca^{2+}-ATPase reaction sequence.

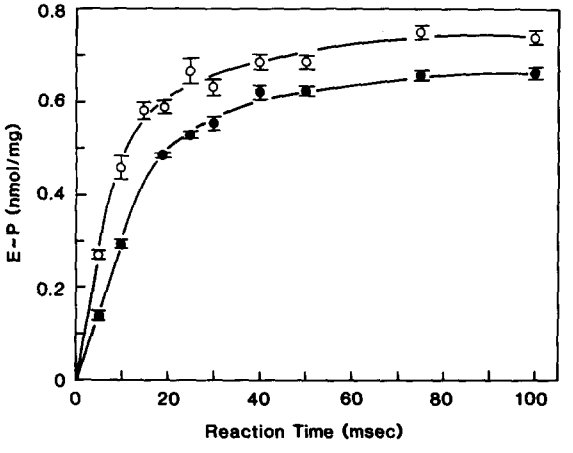

Fig. 1. Effect of protein kinase-dependent phosphorylation of skeletal sarcoplasmic reticulum on the time course of formation of the phosphorylated enzyme intermediate (E ~ P) in calcium-free sarcoplasmic reticulum (E). Skeletal sarcoplasmic reticulum was preincubated with cAMP and cAMP-dependent protein kinase in the presence (open symbols) or absence (closed symbols) of ATP as described under Methods. The sarcoplasmic reticulum was then centrifuged, washed and added to the standard vehicle solution containing 200 μM EGTA. The ATPase reaction was started by the addition of 200 μM [γ-^{32}P]ATP (final 100 μM) and 152 μM of $CaCl_2$ (final 76 μM) which gave 2 μM free Ca^{2+}. Sarcoplasmic reticulum 0.7 mg/ml final concentration. The data represent the mean $\pm$S.E. for four determinations. The Ca^{2+} ATPase activity assayed as described under Methods was 2.8 μmol P_i/mg per min and 3.4 μmol P_i/mg per min for control and phosphorylated sarcoplasmic reticulum, respectively.

Effect of phosphorylation by protein kinase on formation of the phosphorylated intermediate (E ~ P) of Ca^{2+}-free vesicles

The effect of phosphorylation by protein kinase on formation of the phosphorylated intermediate of Ca^{2+}-ATPase was studied on three different sarcoplasmic reticulum preparations. Representative results on the time course (5–100 ms) of E ~ P formation of one of the sarcoplasmic reticulum preparations are shown in Fig. 1. Phosphorylation of skeletal sarcoplasmic reticulum by protein kinase resulted in stimulation of both the apparent initial rate of E ~ P formation and the steady-state level of E ~ P in Ca^{2+}-free (preincubated with EGTA) microsomes (Fig. 1). The maximal (at 100 ms) level of E ~ P formed at 2 μM free Ca^{2+} and 100 μM ATP was 0.65 $\pm$ 0.01 and 0.75 $\pm$ 0.015 nmol/mg of protein for control and phosphorylated skeletal sarcoplasmic reticulum preparations, respectively ($P = 0.005$). The half-time for approach to maximal E ~ P level ($t_{1/2}$) was markedly shortened (7 ms and 11 ms for phosphorylated and control sarcoplasmic reticulum, respectively) by protein kinase (Fig. 1).

Protein kinase-dependent phosphorylation of sarcoplasmic reticulum also resulted in stimulation of the E ~ P levels in the initial phase (0–5 ms) of the reaction (Fig. 2). The initial rates were dependent upon Ca^{2+} concentrations between 0.25–7 μM and exhibited increases upon protein kinase-dependent phosphorylation of sarcoplasmic reticulum. The effect of protein kinase was evident within a wide range of ionized Ca^{2+} (0.25–10 μM), but was mostly sigificant ($P \leqslant 0.01$) at [Ca^{2+}] $\geqslant$ 2 μM.

Effect of phosphorylation by protein kinase on formation of the phosphorylated intermediate (E ~ P) of Ca^{2+}-ATPase in Ca^{2+}-bound vesicles

Formation of the phosphorylated intermediate of Ca^{2+}-ATPase was also studied using Ca^{2+}-bound (preincubated with calcium/EGTA) microsomes, in which E ~ P formation occurs more rapidly than in Ca^{2+}-free sarcoplasmic reticulum [24]. Phosphorylation by protein kinase resulted in a stimulation of E ~ P levels assayed at 2 μM free Ca^{2+} using four different sarcoplasmic reticulum

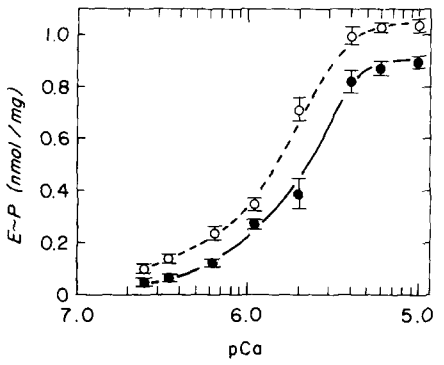

Fig. 2. Effect of protein kinase-dependent phosphorylation on the Ca^{2+}-dependence profile of E ~ P levels at 5 ms in Ca^{2+}-free sarcoplasmic reticulum (E). Skeletal sarcoplasmic reticulum (a different preparation to that used in Fig. 1) was preincubated in the presence (open symbols) or absence (closed symbols) of ATP and treated as described in Fig. 1. The ATPase reaction was started by the addition of 200 μM [γ-^{32}P]ATP (final 100 μM) and various [$CaCl_2$] which gave various free Ca^{2+} concentrations. Sarcoplasmic reticulum, 0.5 mg/ml final concentration. The data represent the mean ±S.E. for four determinations. The Ca^{2+}-ATPase activity was 3 μmol P_i/mg per min for control and 5.45 μmol P_i/mg per min for phosphorylated sarcoplasmic reticulum.

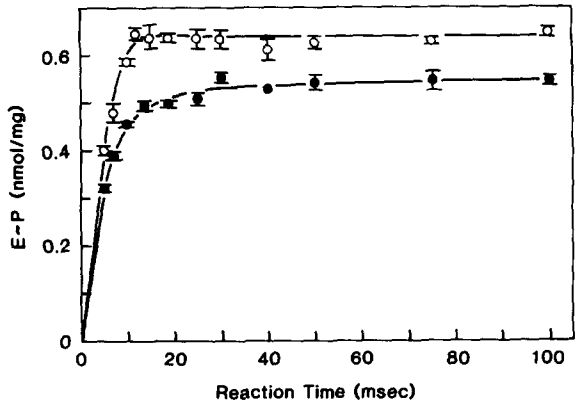

Fig. 3. Effect of protein kinase dependent phosphorylation of skeletal sarcoplasmic reticulum on the time course of E ~ P formation in Ca^{2+}-bound sarcoplasmic reticulum (E·Ca_2). Skeletal sarcoplasmic reticulum was preincubated in the presence (open symbols) or absence (closed symbols) of ATP as described under Methods. Reaction mixtures were centrifuged, sarcoplasmic reticulum were washed and added to the standard vehicle solution containing 100 μM EGTA and 76 μM $CaCl_2$ (2 μM free Ca^{2+}). The ATPase reaction was started by the addition of [γ-^{32}P]ATP (final 100 μM) containing 2 μM free Ca^{2+}. Sarcoplasmic reticulum, 0.65 mg/ml final concentration. The data represent the mean ±S.E. for four determinations. The Ca^{2+} ATPase activity was 2.9 μmol P_i/mg per min and 3.4 μmol P_i/mg per min for control and phosphorylated sarcoplasmic reticulum, respectively.

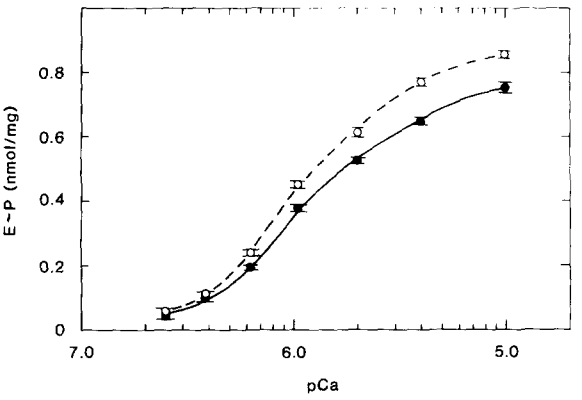

Fig. 4. Effect of protein kinase-dependent phosphorylation on the Ca^{2+}-dependence profile of E ~ P levels at 5 ms in Ca^{2+}-bound sarcoplasmic reticulum (E·Ca_2). Skeletal sarcoplasmic reticulum (a different preparation to that used in Fig. 3) were preincubated in the presence (open symbols) or absence (closed symbols) of ATP and treated as described in Fig. 3. The sarcoplasmic reticulum preparations were added to the standard vehicle solution containing 100 μM EGTA and various [$CaCl_2$]. The ATPase reaction was started by the addition of 200 μM [γ-^{32}P]ATP (100 μM final) and 4 mM EGTA (2 mM final). Sarcoplasmic reticulum, 0.5 mg/ml final concentration. The data represent the mean ±S.E. for four determinations. The Ca^{2+} ATPase activity was 4.3 μmol P_i/mg per min for control sarcoplasmic reticulum and 4.9 μmol P_i/mg per min for phosphorylated sarcoplasmic reticulum.

preparations. Representative results on the time course (5–100 ms) of E ~ P formation of one of the sarcoplasmic reticulum preparations are shown in Fig. 3. The maximal (at 100 ms) level of E ~ P was 0.55 ± 0.006 and 0.65 ± 0.01 nmol/mg protein for control and phosphorylated sarcoplasmic reticulum, respectively ($p = 0.001$). The time at which half of the maximal amount of E ~ P was obtained ($t_{1/2}$) was approximately the same for control and phosphorylated sarcoplasmic reticulum (~ 4 ms).

The effect of protein kinase was also studied on the initial phase of E ~ P formation using Ca^{2+}-bound sarcoplasmic reticulum (Fig. 4). The initial rates were dependent upon Ca^{2+} concentrations between 0.15 and 10 μM. Phosphorylation by protein kinase resulted in significant ($P \leqslant 0.0005$) stimulation of E ~ P levels at Ca^{2+} concentrations between 0.6–10 μM (Fig. 4).

Effect of phosphorylation by protein kinase on decomposition of the phosphorylated intermediate (E ~ P) of Ca^{2+}-ATPase

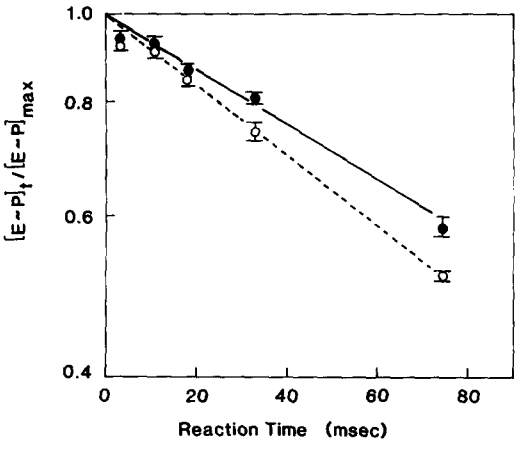

Fig. 5. Effect of protein kinase-dependent phosphorylation of skeletal sarcoplasmic reticulum on the time course of decomposition of the phosphorylated enzyme intermediate, E ~ P, of the Ca^{2+}-ATPase. Skeletal sarcoplasmic reticulum preincubated in the presence (open symbols) and absence (closed symbols) of ATP was assayed for E ~ P formation as described in Fig. 3. Reaction conditions were 0.7 mg sarcoplasmic reticulum/ml, 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 ± S.E. for four determinations. The Ca^{2+} ATPase activity was 3.2 μmol P_i/mg per min and 3.9 μmol P_i/mg per min for control and phosphorylated sarcoplasmic reticulum, respectively.

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% [28]. The time courses of decomposition for control and phosphorylated skeletal sarcoplasmic reticulum were obtained for four different sarcoplasmic reticulum preparations and representative results are shown in Fig. 5. Phosphorylation by protein kinase resulted in stimulation of the initial rate of E ~ P decomposition. The rate constants of E ~ P decomposition were 7.2 ± 0.45 s^{-1} for control and 8.8 ± 0.36 s^{-1} for phosphorylated sarcoplasmic reticulum. These rate constants are significantly different ($P = 0.008$). The decomposition rate constant of untreated sarcoplasmic reticulum (same sarcoplasmic reticulum preparation, kept at 0°C) was not significant different from that of the control sarcoplasmic reticulum.

Discussion

Several phosphoproteins have been identified associated with skeletal sarcoplasmic reticulum vesicles although their role has not yet been clearly defined. In heavy sarcoplasmic reticulum vesicles, derived from the terminal cisternae, phosphoproteins with M_r 47000 and 57000 [19] as well as phosphoproteins with M_r 20000, 40000 and 64000 [14] have been identified. Recently, protein substrates for an endogenous calmodulin-dependent protein kinase associated with fast skeletal sarcoplasmic reticulum have been identified and their M_r ranges between 20000 and 90000 [15–17]. Although Kirchberger and Antonetz [17] and Campbell and MacLennan [15] suggest a role for the calmodulin-dependent phosphorylation of skeletal sarcoplasmic reticulum proteins in regulating Ca^{2+} transport, Chiesi and Carafoli [16] claim that calmodulin-dependent phosphorylation plays no role in the regulation of the active Ca^{2+} uptake reaction. Thus, the effects of calmodulin-dependent phosphorylation of skeletal sarcoplasmic reticulum vesicles have not been established.

We have previously shown that skeletal sarcoplasmic reticulum contains a 100 kDa phosphoprotein, and that this phosphorylation may be related to a marked increase in the overall rates of ATP hydrolysis and Ca^{2+} uptake [13]. Phosphorylation of the 100 kDa substrate occurs by endogenous protein kinase, which is not stimulated by cAMP or calmodulin, and by exogenous cAMP-dependent protein kinase. The present study was undertaken to elucidate the mechanism by which phosphorylation of skeletal SR regulates the Ca^{2+}-ATPase activity. Since steady state results represent the composite of the several steps in the Ca^{2+}-ATPase reaction sequence (Eqn. 1), we examined the effect of phosphorylation on the individual steps to determine which ones were affected. We found that phosphorylation (stable phosphoester) of skeletal sarcoplasmic reticulum by protein kinase appeared to be associated with stimulation of the apparent initial rates and levels of the phosphorylated intermediate (E ~ P) of the Ca^{2+}-ATPase in a manner similar to that found for cardiac sarcoplasmic reticulum [8,9]. Starting with Ca^{2+}-free enzyme (E) an increase in the apparent intial rates was observed within a wide

wide range of Ca^{2+} concentrations. Also the apparent half-time ($t_{1/2}$) of E ~ P formation was shortened by protein kinase dependent phosphorylation (11 ms and 7 ms for control and phosphorylated sarcoplasmic reticulum, respectively). Starting with Ca^{2+}-bound enzyme ($E \cdot Ca_2$) an increase in the apparent initial rates of E ~ P formation was also observed over a wide range of Ca^{2+} concentrations. Due to the rapidity of E ~ P formation in this case it was difficult to assess whether the apparent half-time ($t_{1/2}$) was affected by protein kinase. The increase in the initial rate of E ~ P formed may be accounted for by an increase in Ca^{2+} or ATP binding and/or subsequent steps of E ~ P formation. Furthermore, we previously showed that the Ca^{2+}-ATPase from skeletal and cardiac microsomes is present in two forms (E′ and E) and that the rate of conversion of E′ to E is much slower than that for calcium binding [24]:

$$E' \leftrightarrow E + 2\,Ca^{2+} \leftrightarrow E \cdot Ca_2 \qquad (2)$$

Therefore, the step of conversion of E′ to E may be one of the major steps affected by protein kinase in both cardiac and skeletal sarcoplasmic reticulum.

Phosphorylation of rabbit skeletal sarcoplasmic reticulum by protein kinase also stimulated the initial phase (0–73 ms) of E ~ P decomposition ($K_d = 7.2$ and 8.8 s^{-1} for control and phosphorylated sarcoplasmic reticulum, respectively). E ~ P decomposition is thought to be one of the slow steps in the Ca^{2+}-ATPase reaction sequence [29] and therefore may regulate the overall enzymatic activity. We have shown in the present study that the initial rates of both E ~ P formation and decomposition are regulated by protein kinase. These findings suggest that the Ca^{2+}-ATPase itself is directly affected by protein kinase mediated phosphorylation of sarcoplasmic reticulum membranes. Enhancement of any of the steps in Eqn. 1 could account for the observed stimulation of the calcium pump previously reported [13].

The alterations in the Ca^{2+}-ATPase reaction steps which we report here do not represent any nonspecific changes occurring during exposure of sarcoplasmic reticulum to different reactants (ions, cAMP or protein kinase) in the preincubation assay or during washing of the sarcoplasmic reticulum. Control sarcoplasmic reticulum was preincubated under the same conditions as phosphorylated sarcoplasmic reticulum but in the absence of ATP to prevent protein kinase-mediated phosphorylation. Also, the phosphorylated and nonphosphorylated states of skeletal sarcoplasmic reticulum 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 sarcoplasmic reticulum activity to changes associated with protein kinase-dependent phosphorylation of sarcoplasmic reticulum. While the mechanism for these alterations is not known, one of the most interesting findings in this study is that phosphorylation of either a 22 kDa substrate in cardiac muscle or a 100 kDa substrate in skeletal sarcoplasmic reticulum controls Ca^{2+} transport by regulating the same individual steps in the Ca^{2+}-ATPase reaction sequence of both muscles. Therefore, it is probably not essential to have a specific phosphoprotein present in sarcoplasmic reticulum but the localization of this protein in the microenvironment of the Ca^{2+}-ATPase may be important for the effects of its phosphorylation on the Ca^{2+}-ATPase activity. However, proof that phosphorylation of either skeletal or cardiac sarcoplasmic reticulum regulates calcium transport must await isolation of protein substrates and determination of the effect of their phosphorylation in a reconstituted calcium pump. Ultimately, phosphorylation of sarcoplasmic reticulum proteins must be demonstrated in vivo and a correlation between the degree of phosphorylation and contractility must be established.

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