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PHOSPHORYLATION OF A 100 000 DALTON COMPONENT AND ITS RELATIONSHIP TO CALCIUM TRANSPORT IN SARCOPLASMIC RETICULUM FROM RABBIT SKELETAL MUSCLE

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Summary

Sarcoplasmic reticulum from rabbit fast skeletal muscle contains intrinsic protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) and a substrate. The protein kinase activity was Mg^{2+} dependent and could also phosphorylate exogenous protein substrates. Autophosphorylation of sarcoplasmic reticulum vesicles was not stimulated by cyclic AMP, neither was it inhibited by the heat-stable protein kinase inhibitor protein. The phosphorylated membranes had the characteristics of a protein with a phosphoester bond. An average of 73 pmol P_i/mg protein were incorporated in 10 min at 30°C. Addition of exogenous cyclic AMP-dependent protein kinase increased the endogenous level of phosphorylation by 25—100%. Sarcoplasmic reticulum membrane phosphorylation, mediated by either endogenous cyclic AMP-independent or exogenous cyclic AMP-dependent protein kinase, occurred on a 100 000 dalton protein and both enzyme activities resulted in enhanced calcium uptake and Ca^{2+}-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3), in a manner similar to cardiac microsomal preparations.

Regulation of Ca^{2+} transport in skeletal sarcoplasmic reticulum may be mediated by phosphorylation of a 100 000 dalton component of these membranes.

Introduction

Protein kinases exist in a variety of mammalian tissues [1] including skeletal muscle, from which these enzymes have been purified and characterized [2—5].

The substrate specificity of the muscle protein kinases like that of other protein kinases is broad and they can catalyze the phosphorylation of several protein substrates. Studies on the purification and characterization of protein kinases are rapidly expanding but little is known about their natural protein substrates. Prompted by our previous demonstration of intrinsic protein kinase activity in skeletal sarcoplasmic reticulum preparations [6] and the recent demonstration by Fabiato and Fabiato [7] that intact fibers of cat fast skeletal muscle respond to cyclic AMP probably at the sarcoplasmic reticulum level, we have attempted to identify the endogenous substrate of the skeletal sarcoplasmic reticulum protein kinase. The results indicate that a single membrane protein of molecular weight 100 000 serves as substrate for both endogenous (cyclic AMP-independent) and exogenous (cyclic AMP-dependent) protein kinases and phosphorylation of this protein enhances energy-dependent Ca^{2+} uptake and Ca^{2+}-ATPase activity.

Experimental procedure

Materials. Biochemical reagents including beef heart cyclic AMP-dependent protein kinase were purchased from Sigma Chemical Co., St. Louis, MO. All chemicals were of 'chemical pure grade'. Adenosine 5'-[γ-^{32}P]triphosphate, ammonium salt (10—40 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Heat-stable protein kinase inhibitor protein, prepared by the method of Ashby and Walsh [8], was a gift from Mr. M. Laks of Northwestern University Medical School, to whom we are most grateful.

Preparation of sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles from rabbit skeletal muscle were prepared by the procedure of Harigaya and Schwartz [9] with some modifications as recently described [10]. The extent of mitochondrial contamination of the microsomal fraction was checked by determination of cytochrome *c* oxidase activity [11]. The activity per mg of protein in this fraction was less than 2% of that in the mitochondrial fraction (5000 × *g* pellet). Ouabain-sensitive ($Na^+ + K^+$)-ATPase and [^{3}H]-ouabain binding, enzymatic markers of sarcolemma [12], were less than 0.5% (0.005 mol active ($Na^+ + K^+$)-ATPase/mol active Ca^{2+}-ATPase) of the total ATPase of these membrane preparations.

Phosphorylation of sarcoplasmic reticulum vesicles. Phosphorylation reactions were carried out in a volume of 0.2 ml at pH 6.8 in the presence of 50 mM phosphate buffer, 10 mM $MgCl_2$, 10 mM NaF, 0.5 mM EGTA, 200 μg of sarcoplasmic reticulum protein and 500 μM [γ-^{32}P]ATP (120—200 cpm/pmol) for 10 min at 30°C. The reaction was terminated by the addition of 2 ml of 7% ice-cold $HClO_4$ containing 7% poly(phosphate) and 0.5 mg of carrier skeletal sarcoplasmic reticulum protein was added. The samples were centrifuged and the pellet was washed three times with 7% $HClO_4$ containing 7% poly(phosphoric acid). The final pellet was dissolved in 0.5 ml of 10 mM NaOH containing 0.1 mM $Na_2PO_4 \cdot H_2O$; 10 ml of Aquasol II (New England Nuclear) was added and radioactivity was determined in a model 3320 Packard Tri-Carb liquid scintillation spectrometer.

In experiments in which protein kinase-catalyzed sarcoplasmic reticulum phosphorylation was correlated with protein kinase-stimulated calcium uptake

and calcium-activated ATPase activity, sarcoplasmic reticulum vesicles were preincubated in the same reaction medium described above for measuring sarcoplasmic reticulum phosphorylation, except that unlabeled ATP was used and NaF was omitted. Control vesicles were also incubated under identical conditions without ATP. Protein kinase (Sigma) and cyclic AMP, when added, were 0.1 mg/ml and 1 μM, respectively. Cyclic AMP-dependent protein kinase inhibitor, when present, was 10 μg/ml, a concentration which inhibited the cyclic AMP-dependent protein kinase activity (Sigma) by 85% using histone as substrate. After 10 min of incubation at 30°C, the mixture was centrifuged at 105 000 $\times g$ for 30 min and the pellet was homogenized gently in ice-cold 100 mM KCl, 20 mM Tris/maleate (pH 6.8). Sarcoplasmic reticulum protein recovery after this procedure was 90% and did not vary with the nature of the pretreatment. Assays for calcium uptake and Ca^{2+}-ATPase were subsequently carried out as described below.

Release of [^{32}P]orthophosphate from labeled sarcoplasmic reticulum. 200 μg of washed phosphorylated sarcoplasmic reticulum containing about 700—1200 cpm of ^{32}P were suspended and incubated for 10 min in 1 ml of the following solutions: (a) 10% (w/v) trichloroacetic acid at 0°C; (b) 10% (w/v) trichloroacetic acid at 90°C; (c) C_2H_5OH/ether (1 : 1, v/v) at 30°C; (d) 0.5 N NaOH at 90°C; (e) 0.8 M hydroxylamine/0.01 M sodium acetate buffer, pH 5.3, at 30°C, and (f) 0.8 M NaCl/0.01 M sodium acetate buffer, pH 5.3 at 30°C. Reactions were terminated by addition of 1 ml of ice-cold 50% trichloroacetic acid. The samples were centrifuged at 1000 rev./min for 5 min and radioactivity contained in the pellets and the supernatant fluid was determined as above.

Polyacrylamide gel electrophoresis of phosphorylated membranes. Sarcoplasmic reticulum (2 mg) was incubated with [γ-^{32}P]ATP (200—1000 cpm/pmol) in a total volume of 2 ml under the conditions of the phosphorylation assay as described above. After 10 min incubation at 30°C, the reaction mixture was cooled on ice and subsequently subjected to chromatography on Sephadex G-50 (column dimensions 1.8 $\times$ 11 cm) at 4°C to separate ^{32}P-labeled sarcoplasmic reticulum from the phosphorylation assay reactants.

Polyacrylamide gel electrophoresis of ^{32}P-labeled sarcoplasmic reticulum under denaturing conditions was carried out at pH 7.1 in the presence of 0.1% sodium dodecyl sulfate according to the method of Maizel [13] and Weber and Osborn [14]. The molecular weight of the radioactive peak was determined by utilizing appropriate markers and constructing a relative mobility curve; standards used were phosphorylase *a* (M_r 100 000), human serum albumin (M_r 66 000), DNAase 1 (M_r 31 000) and egg-white lysozyme (M_r 14 300).

Assay for calcium uptake. Oxalate-facilitated calcium uptake was determined at 25°C in 40 mM Tris/maleate (pH 6.8), 100 mM KCl, 3 mM $MgCl_2$, 5 mM ATP, 5 mM sodium azide, 2.5 mM Tris/oxalate and a $^{45}Ca^{2+}$/EGTA buffer system. A computer program was used to calculate the total calcium added to 100 μM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) in order to obtain various Ca^{2+} concentrations at pH 6.8 [15]. Final sarcoplasmic reticulum concentrations were 50 μg/ml. The reaction was initiated by adding ATP and was terminated by passing aliquots of the reaction mixture through a type HA Millipore filter (0.45 μm average pore diameter) [16]. The filters were washed with 20 ml containing 20 mM Tris/maleate (pH 6.8), 0.1

M KCl and 4 mM EGTA, dried and counted for radioactivity in Aquasol (New England Nuclear).

ATPase assay. The ATPase activity of sarcoplasmic reticulum was determined at 25°C in a reaction mixture identical to the one used for assaying calcium uptake. Reaction mixtures were equilibrated at 25°C for 5 min after which the reactions were started by the addition of ATP. To determine 'basic' (Ca^{2+}-independent) ATPase activity, reactions were carried out in the presence of 0.5 mM EGTA instead of calcium/EGTA buffer. At various time intervals, the reactions were terminated by addition of an equal volume of 10% (w/v) trichloroacetic acid. After centrifugation (1000 × *g*, 5 min at 4°C), the amount of P_i in 1 ml aliquot of the supernatant was determined by mixing it with 1 ml of 1.25% ammonium molybdate in 1 N H_2SO_4 containing 50 mg $FeSO_4$. After 30 min the samples were read at 700 nm.

Ca^{2+}-activated ATPase activity was determined by subtracting the 'basic' ATPase activity from the rate of P_i liberation at each Ca^{2+} concentration.

Calcium-activated ATPase activity and calcium uptake velocity were calculated from the initial phase of the reactions when the time course of the reactions was linear.

Results

Phosphate incorporation into sarcoplasmic reticulum was linear for less than 1 min at 30°C although it increased up to 10 min (Fig. 1). The non-linearity of the incorporation of phosphate was due to depletion of ATP in the reaction mixture. Addition of extra ATP resulted in restoration of the linearity (data not shown). An incubation time of 10 min at 30°C was chosen for assaying sarcoplasmic reticulum protein kinase activity. The sarcoplasmic reticulum membrane and the reactants of the protein kinase assay were incubated for 10 min at 30°C before the addition of [γ-^{32}P]ATP. When preincubation was omitted, only 40% of the enzymatic activity was observed. When NaF was omitted during the preincubation period, 75% of the total protein kinase activity was observed.

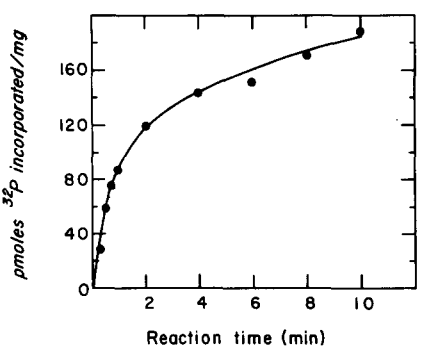

Fig. 1. Time course of skeletal sarcoplasmic reticulum autophosphorylation. Skeletal sarcoplasmic reticulum (200 μg) was incubated in a total reaction volume of 0.2 ml of phosphorylation assay buffer at 30° C. The values shown are the arithmetic means of three determinations.

Under standard assay conditions phosphorylation of endogenous substrate increased with increasing amounts of sarcoplasmic reticulum protein up to 1 mg/ml reaction mixture. The total amount of [^{32}P]phosphate that was incorporated per mg of sarcoplasmic reticulum protein varied (50—145 pmol P_i/mg protein) with different sarcoplasmic reticulum preparations. This variation did not appear to be due to selective proteolysis occurring either during isolation of sarcoplasmic reticulum or during the phosphorylation assay (see below). However, the observed variation may be related to the fact that sarcoplasmic reticulum protein(s) may be partially phosphorylated in vivo or may have undergone phosphorylative modification by protein kinases and phosphatases during the isolation of the sarcoplasmic reticulum preparations. An average value of 73 pmol P_i/mg sarcoplasmic reticulum protein at saturation levels was obtained for twelve different preparations.

Substrate specificity

The relative substrate specificity of the protein kinase was determined using a number of different phosphate acceptor proteins. The protein kinase exhibited a preference for the basic proteins as substrates rather than the acidic proteins (Table I).

Reaction requirements

The effects of various cofactors on the skeletal sarcoplasmic reticulum protein kinase activity are shown in Table II. The phosphorylation reaction showed an absolute requirement for Mg^{2+} with maximal stimulation at 10—20 mM and a requirement for NaF with maximal stimulation at 10 mM. The fluoride stimulation was not due to inhibition of an endogenous phosphatase which was active in the phosphorylation assay. Incubation of ^{32}P-labeled sarcoplasmic reticulum in the phosphorylation reaction mixture, in the absence of NaF, did not result in any detectable loss of radioactive phosphate from the ^{32}P-labeled sarcoplasmic reticulum. However, NaF inhibited the Ca^{2+}-ATPase

TABLE 1

PHOSPHORYLATION OF ENDOGENOUS AND EXOGENEOUS SUBSTRATES BY SARCOPLASMIC RETICULUM PROTEIN KINASE

Sarcoplasmic reticulum membrane protein kinase activity was measured and the protein substrates were adjusted to the same concentration on a mg/ml basis. 100 μg of substrate was used per assay. The dephosphorylated forms of phosvitin and casein were used [8]. The values shown are the arithmetic means of three determinations.

Added protein substrate	Protein kinase activity (pmol/mg per 10 min)	Activity *
None	115	100
Total histone	151	131
Protamine sulfate	150	130
Histone: arginine rich	142	123
Histone: lysine rich	116	101
Casein	108	94
Phosvitin	102	89

* Relative to control (100%).

TABLE II

REACTION REQUIREMENTS FOR SARCOPLASMIC RETICULUM AUTOPHOSPHORYLATION

Rabbit skeletal sarcoplasmic reticulum phosphorylation was measured with the indicated reactants deleted from or added to the reaction mixture. All reaction volumes were identical. The values shown are the arithmetic means of three determinations.

Deletions or additions of reactants	Protein kinase activity (pmol/mg per 10 min)	Activity *
None	80	100
$-MgCl_2$	7	9
—NaF	14	18
—EGTA	50	62
+Theophylline	77	96
+Dithiothreitol	73	91

* Relative to control (100%).

activity of the sarcoplasmic reticulum (unpublished data). Addition of 2 mM theophylline or 1 mM dithiothreitol did not affect the protein kinase activity. Inclusion of EGTA stimulated phosphate incorporation. The presence of Ca^{2+} concentration higher than 10^{-8} M was inhibitory in the phosphorylation reaction and maximum inhibition (80%) was obtained at $5 \cdot 10^{-6}$ M Ca^{2+} (Fig. 2) indicating that the sarcoplasmic reticulum protein kinase was not phosphorylase kinase. The inhibition by 10^{-6} M Ca^{2+} did not appear to be due to activation of a Ca^{2+}-dependent phosphatase activity in sarcoplasmic reticulum. When sarcoplasmic reticulum was phosphorylated in the presence of 10^{-8} M Ca^{2+} and the calcium concentration in the reaction mixture was subsequently raised to

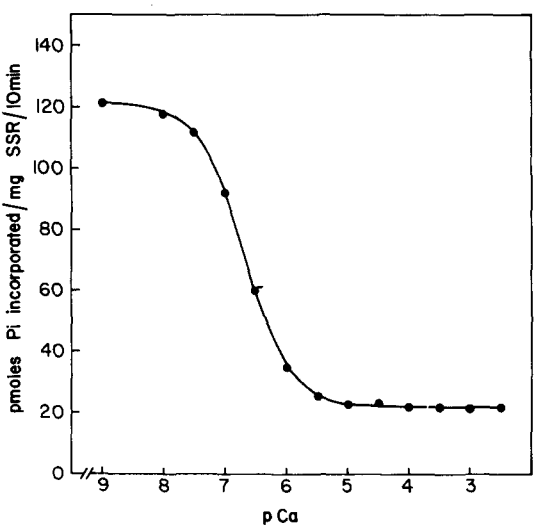

Fig. 2. Ca^{2+} dependence of skeletal sarcoplasmic reticulum autophosphorylation. Skeletal sarcoplasmic reticulum was phosphorylated in the presence of various Ca^{2+} concentrations indicated on the abscissae. Ca^{2+} concentrations were maintained by calcium/EGTA buffers containing 2 mM EGTA and various amounts of $CaCl_2$ calculated by a computer program [15]. Calcium contamination by sarcoplasmic reticulum was determined by atomic absorption spectrometry (Perkin Elmer 290). The values shown are the arithmetic means of three determinations.

10^{-6} M there was no detectable dephosphorylation of the sarcoplasmic reticulum. Maximum sarcoplasmic reticulum protein kinase activity occurred at pH 7.0 and the pH curve showed a rather steep maximum.

Cyclic AMP and cyclic GMP independency of skeletal sarcoplasmic reticulum protein kinase activity

Cyclic AMP or cyclic GMP concentrations between $1 \cdot 10^{-7}$ and $1 \cdot 10^{-3}$ M did not stimulate phosphate incorporation into sarcoplasmic reticulum above the basal level. The sarcoplasmic reticulum protein kinase activity was also assayed with increasing amounts (15—30 μg/200 μl assay mixture) of the heat-stable protein kinase inhibitor protein [17,18]. Saturating concentrations of the inhibitor, which inhibit the beef heart cyclic AMP-dependent protein kinase (Sigma) by 85%, had no effect on the sarcoplasmic reticulum protein kinase activity (Table III).

Phosphorylation of skeletal sarcoplasmic reticulum by exogenous beef heart cyclic AMP-dependent protein kinase

Sarcoplasmic reticulum phosphorylation was stimulated (25—100%) in the presence of cyclic AMP and exogenous cyclic AMP-dependent protein kinase. Phosphorylation increased with increasing protein kinase concentration until the protein kinase concentration reached about 0.1 mg/ml (data not shown). Subsequent experiments were carried out at a protein kinase concentration of 0.1 mg/ml at a ratio of sarcoplasmic reticulum protein to protein kinase of 10 : 1. The protein kinase-stimulated phosphorylation was inhibited by the protein kinase inhibitor (Table III). The data shown in Table III have been corrected for the degree of autophosphorylation of the cyclic AMP-dependent protein kinase determined under identical experimental conditions.

Characterization of phosphorylated membrane component

Trichloroacetic acid-precipitated sarcoplasmic reticulum membranes which had been phosphorylated by endogenous protein kinase were resistant to extraction by C_2H_5OH/ether (1 : 1, v/v) or by hot trichloroacetic acid, ruling out

TABLE III

THE EFFECTS OF PROTEIN KINASE AND THE HEAT-STABLE PROTEIN KINASE INHIBITOR PROTEIN ON SARCOPLASMIC RETICULUM PHOSPHORYLATION

2 μg of protein kinase inhibitor and/or 20 μg of beef heart cyclic AMP-dependent protein kinase were incubated at 30°C in the presence of 1 μM cyclic AMP in a total volume of 0.13 ml of protein kinase assay buffer. After 3 min of incubation time, 200 μg of sarcoplasmic reticulum protein in 20 μl were added and incubation continued for an additional 10 min. Reactions were started by the addition of 50 μl containing 100 nmol [γ-^{32}P]ATP. The values shown are the arithmetic means of three determinations.

Addition	Protein kinase activity (pmol/mg per 10 min)	Activity *
Control	78	100
Control + protein kinase inhibitor	79	101
Control + protein kinase	117	150
Control + protein kinase + protein kinase inhibitor	82	105

* Relative to control (100%).

lipid or nucleic acid as the phosphorylated component (Table IV). The [^{32}P]-phosphate was hydrolyzed by hot 0.5 N NaOH, but it was resistant to incubation with 0.8 M hydroxylamine (Table IV) indicating the presence of phosphoester bonds [19].

Gel electrophoresis of phosphorylated sarcoplasmic reticulum membranes

Sarcoplasmic reticulum membranes were incubated with [γ-^{32}P]ATP under conditions of optimum phosphorylation and subsequently subjected to chomatography on Sephadex G-50. Analysis of the ^{32}P-labeled sarcoplasmic reticulum eluted from Sephadex G-50 by electrophoresis on polyacrylamide gels under denaturing conditions revealed a number of protein peaks, the most prominent of which had a molecular weight of 100 000 (Fig. 3A). When smaller amounts of sarcoplasmic reticulum protein were electrophoresed on polyacrylamide gels under identical conditions, the 100 000 molecular weight protein peak was resolved into two protein peaks (Fig. 3B). Using both 7% acrylamide gels (data not shown) and mixed gels (5% acrylamide in the upper half and 10% acrylamide in the lower half of the gel) the 100 000 molecular weight protein was identified as the [^{32}P]phosphate acceptor protein.

When sarcoplasmic reticulum was phosphorylated in the presence of cyclic AMP and beef heart cyclic AMP-dependent protein kinase, [^{32}P]phosphate incorporation into the 100 000 dalton protein was increased up to 2-fold over the level of phosphorylation by endogenous protein kinase. The 100 000 dalton protein was the only ^{32}P-labeled protein observed in skeletal sarcoplasmic reticulum phosphorylated by either endogenous or exogenous protein kinase even when [γ-^{32}P]ATP of high specific activity (1000 cpm/pmol) was used. The amounts of the 100 000 dalton protein relative to the other proteins present in sarcoplasmic reticulum did not vary when 0.5 mM phenyl methylsulfonyl fluoride was present during isolation of sarcoplasmic reticulum or during preincubation (0—30 min at 30°C) in the phosphorylation assay medium. In addition, the levels of phosphate incorporated into the 100 000 dalton protein were not affected by phenyl methylsulfonyl fluoride addition. Furthermore, phosphorylation of sarcoplasmic reticulum by exogenous protein

TABLE IV

CHARACTERIZATION OF PHOSPHORYLATED SARCOPLASMIC RETICULUM MEMBRANES

The phosphorylated sarcoplasmic reticulum membranes were acid precipitated, washed three times and subjected to the treatments indicated, each for 10 min. Recovery of [^{32}P]phosphate in samples maintained on ice in 10% trichloroacetic acid was taken as 100%. The values shown are the arithmetic means of three determinations.

Treatment	Phosphorylation	
	pmol P_i/mg per 10 min	%
1. None (control = 10% trichloroacetic acid at 0°C)	112	100
2. Trichloroacetic acid, 10%, 90°C	86	77
3. C_2H_5OH/ether (1 : 1, v/v) 30°C	110	98
4. NaOH, 0.5 N, 90°C	0	0
5. Hydroxylamine, 0.8 M, 30°C	125	103
6. NaCl, 0.8 M, 30°C	109	97

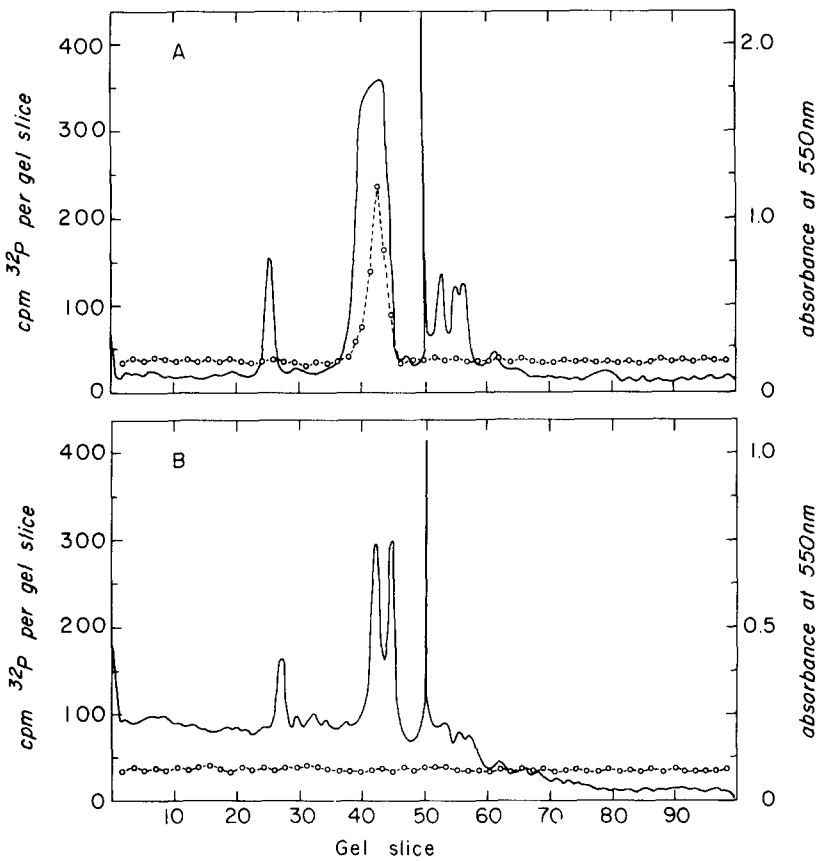

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of phosphorylated skeletal sarcoplasmic reticulum. ^{32}P-labeled sarcoplasmic reticulum obtained after Sephadex G-50 chromatography was applied onto mixed polyacrylamide gels (dimensions 0.6 × 10 cm) consisting of 5% acrylamide in the upper half and 10% acrylamide in the lower half of the gel containing 0.1% sodium dodecyl sulfate. Protein samples were prepared for electrophoresis by incubation at 37°C for 20 min in 10 mM sodium phosphate buffer, pH 7.1, containing 1% mercaptoethanol and 1% sodium dodecyl sulfate. Electrophoresis was carried out at 2.5 m A/gel for 9 h at 25°C. After electrophoresis, the gels were stained, destained, scanned at 550 nm then sliced into 1 mm sections which were counted for the determination of ^{32}P radioactivity. (A) 20 μg of ^{32}P-labeled microsomes; (B) 4 μg of ^{32}P-labeled microsomes; ———, gel scan at 550 nm; o———o, ^{32}P radioactivity.

kinase did not vary when the vesicles were preincubated in the phosphorylation assay medium (0—30 min at 30°C). These results indicate that the phosphorylated protein was not the denatured or proteolytic product of a major sarcoplasmic reticulum protein. When exogenous protein kinase was used, a second peak of radioactivity was also observed and was associated with a 62 000 dalton protein. This may be attributed to the beef heart protein kinase because it was the only radioactive peak found when beef heart protein kinase was phosphorylated in the presence of cyclic AMP under identical conditions.

Effect of phosphorylation on energy-dependent calcium uptake and Ca^{2+}-ATPase activity by skeletal sarcoplasmic reticulum

Phosphorylation of rabbit skeletal sarcoplasmic reticulum by the endogenous

TABLE V

EFFECTS OF ENDOGENOUS AND EXOGENOUS PROTEIN KINASES ON CALCIUM UPTAKE AND Ca^{2+}-ACTIVATED ATPase ACTIVITY OF SARCOPLASMIC RETICULUM

Rabbit skeletal sarcoplasmic reticulum was pretreated under various conditions and subsequently assayed for calcium uptake and calcium-activated ATPase activity in the presence of 1 μM Ca^{2+} (100 μM EGTA, 60.4 μM $CaCl_2$) as described under Experimental procedure. Values are means for six determinations. S.E. are indicated. The stoichiometric ratio was calculated by dividing the rate of calcium uptake by the rate of Ca^{2+}-activated ATPase. PK, protein kinase.

Pretreatment	Calcium uptake (nmol Ca^{2+}/min per mg)	Ca^{2+}-ATPase (nmol P_i/min per mg)	Stoichiometric ratio ($Ca^{2+}t$: P_i)
Control	541 ± 41	340 ± 16	1.59
+ Inhibitor	591 ± 16	338 ± 13	1.74
+ ATP	650 ± 46	406 ± 20	1.6
+ ATP + cyclic AMP	683 ± 28	413 ± 25	1.65
+ ATP + cyclic AMP + inhibitor	618 ± 25	413 ± 25	1.5
+ PK	608 ± 48	380 ± 25	1.6
+ ATP + PK	683 ± 21	415 ± 26	1.64
+ ATP + cyclic AMP + PK	950 ± 51	545 ± 23	1.74
+ ATP + cyclic AMP + boiled PK	683 ± 40	436 ± 20	1.56
+ ATP + cyclic AMP + PK + inhibitor	636 ± 26	433 ± 20	1.47

protein kinase in the presence of ATP resulted in a statistically significant ($P < 0.03$) increase of approx. 18% in the initial rate of calcium uptake and calcium-activated ATPase activity (Table V). The presence of cyclic AMP or the heat-stable protein kinase inhibitor in the preincubation reaction media did not significantly affect the stimulatory effect produced by the endogenous protein kinase in accordance with our observations above (Table III). Pretreatment of sarcoplasmic reticulum with the cyclic AMP-dependent protein kinase and cyclic AMP resulted in a statistically significant ($P < 0.01$) increase of the initial rate of calcium uptake (75.3%, see Table V), and calcium-activated ATPase activity (60.2%, Table V) over that produced by the sarcoplasmic reticulum protein kinase. This stimulatory effect was dependent on the presence of cyclic AMP and it was abolished when boiled protein kinase or the protein kinase inhibitor were used (Table V). Pretreatment of microsomes under various conditions did not alter the stoichiometry of approx. 1.6 mol Ca^{2+} taken up/mol of ATP hydrolyzed (Table V).

Discussion

Phosphorylation of skeletal muscle sarcoplasmic reticulum by endogenous or exogenous protein kinase has been the subject of controversy. We have previously presented evidence that cat skeletal muscle (tibialis, caudofemoralis and soleus) sarcoplasmic reticulum preparations were phosphorylated by endogenous and exogenous protein kinase [6]. Fabiato and Fabiato [7], using skinned fibers from cat caudofemoralis, observed muscle relaxation in the presence of 0.5 mM EGTA and 5 μM cyclic AMP and suggested that the site of the cyclic AMP effect was at the sarcoplasmic reticulum level. Very recently, Carstens and Weller [20] reported phosphorylation of rat skeletal microsomes by an endog-

enous cyclic AMP-dependent protein kinase. On the other hand, Kirchberger and Tada [21] and Caswell et al. [22] were not able to detect phosphorylation of rabbit skeletal muscle microsomes by ATP, skeletal muscle protein kinase and cyclic AMP.

In this report we present evidence that skeletal sarcoplasmic reticulum membranes contain an intrinsic protein kinase activity which can phosphorylate an endogenous membrane component. The chemical stability of the acid-precipitable [^{32}P]phosphate indicates that the phosphate was bound to sarcoplasmic reticulum protein through a phosphoester bond. Addition of exogenous cyclic AMP-dependent protein kinase significantly augmented phosphorylation of skeletal sarcoplasmic reticulum confirming our previous report [6]. We feel that Kirchberger and Tada [21] and Caswell et al. [22] were not able to observe phosphorylation of skeletal microsomes because their phosphorylation assays were not performed under optimum conditions (high specific activity of [γ-^{32}P]ATP, preincubation of membranes at 30°C before starting the reaction, sarcoplasmic reticulum protein concentration up to 1 mg/ml and exogenous protein kinase of high specific activity).

The rabbit skeletal sarcoplasmic reticulum protein kinase activity was found to be inhibited at free calcium concentrations higher than 10^{-8} M indicating that the activity was not phosphorylase kinase [23—25]. In fact, free calcium concentrations higher than $5 \cdot 10^{-6}$ M inhibited the endogenous phosphorylation by 80%. Our data suggest that skeletal sarcoplasmic reticulum phosphorylation may, however, be regulated by calcium and therefore phosphorylation may play an important role during the contraction-relaxation cycle. The sarcoplasmic reticulum protein kinase activity, in contrast to the purified soluble enzymes [2—5], was not inhibited by the heat-stable inhibitor protein. The sarcoplasmic reticulum protein kinase may either be a cyclic AMP-independent protein kinase or the catalytic subunit of a cyclic AMP-dependent holoenzyme [17,18] which is bound to the membrane so that the site of action of the heat-stable inhibitor protein is blocked. Assuming that the sarcoplasmic reticulum protein kinase is the catalytic subunit of a cyclic AMP-dependent holoenzyme, it differs from the soluble cyclic AMP-dependent protein kinases present in rabbit skeletal muscle [2—5] in its substrate specificity (Table I).

Phosphorylation of skeletal sarcoplasmic reticulum membranes by both endogenous and exogenous protein kinases occurred on a 100 000 dalton polypeptide clearly different from the 20 000 dalton protein which has been reported to be phosphorylated in cardiac 'microsomes' [6,26—28]. Wray and Gray [26] reported phosphorylation of a high molecular weight protein in cardiac sarcoplasmic reticulum. However, their phosphoprotein was 130 000 daltons while the one we observe in skeletal sarcoplasmic reticulum is 100 000 daltons. Phosphorylation of the 100 000 dalton protein by endogenous protein kinase stimulated both the rate of calcium transport and Ca^{2+}-ATPase activity. However, phosphorylation by the exogenous cyclic AMP-dependent protein kinase had a more pronounced stimulatory effect on the calcium pump. The stimulatory effect, unlike the one observed by Kirchberger and Tada [21] using high concentrations of protein kinase, was produced by low concentrations of protein kinase, it was dependent on the presence of cyclic AMP and it was abolished in the presence of the protein kinase inhibitor protein. Furthermore,

stimulation of calcium transport and calcium-activated ATPase did not require either protein kinase or cyclic AMP to be present at the time that measurements were made. The stoichiometric ratio of approx. 2 mol of calcium taken up for each mol of ATP hydrolyzed [29—32] was maintained even after phosphorylation of sarcoplasmic reticulum by either endogenous or exogenous protein kinase. This indicates that the overall rate of calcium transport, rather than its efficiency, is enhanced.

In view of the broad and unspecific utilization of substrates by protein kinases in vitro, specificity of phosphorylation is achieved in fully differentiated cells through a stringent compartmentalization of the kinases. Isolated membranes from various tissues contain endogenous protein kinases which phosphorylate membrane proteins and participate in the regulation of membrane ion binding and permeability [33—36]. Skeletal sarcoplasmic reticulum membranes contain an intrinsic protein kinase and a substrate. Phosphorylation of sarcoplasmic reticulum resulted in stimulation of both the initial rate of calcium transport and calcium-activated ATPase activity. Furthermore, phosphorylation of sarcoplasmic reticulum appeared to be regulated by calcium at concentrations similar to those present in the cell during the contraction-relaxation cycle indicating the physiological significance for this regulatory mechanism in skeletal sarcoplasmic reticulum.

The present study suggests that protein kinase-catalyzed phosphorylation of sarcoplasmic reticulum membrane proteins other than 'phospholamban' [26—28] may play a regulatory role in cation transport. However, direct proof that a phosphorylatable membrane protein regulates calcium transport in sarcoplasmic reticulum will have to wait until the phosphoprotein is isolated. The isolated protein must be subsequently added to the corresponding reconstituted calcium pump and the effect of its phosphorylation on calcium transport must be determined. 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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References

1 Kuo, J.F., Krueger, B.K., Sanes, J.R. and Greengard, P. (1970) Biochim. Biophys. Acta 212, 70—91
2 Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1986—1995
3 Corbin, J.D., Brostrom, C.O., King, C.A. and Krebs, E.G. (1972) J. Biol. Chem. 247, 7790—7798
4 Huang, C.L. and Huang, C.H. (1975) Biochemistry 14, 18—24
5 Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) Adv. Cyclic Nucl. Res. 5, 241—251
6 Schwartz, A., Entman, M.L., Kaniike, K., Lane, L.K., Van Winkle, W.B. and Bornet, E.P. (1976) Biochim. Biophys. Acta 426, 57—72.

7 Fabiato, A. and Fabiato, F. (1978) Biochim. Biophys. Acta 539, 253—260
8 Ashby, D.C. and Walsh, D.A. (1974) Methods Enzymol. 38, 350—358
9 Harigaya, S. and Schwartz, A. (1968) Circ. Res. 25, 781—794
10 Sumida, M., Wang, T., Mandel, F., Froehlich, J.P. and Schwartz, A. (1978) J. Biol. Chem. 253, 8772-8777
11 Wharton, D.C. and Tzagoloff, A. (1967) Methods Enzymol. 10, 245—250
12 Wallick, E.T. and Schwartz, A. (1974) J. Biol. Chem. 249, 5141—5147
13 Maizel, J.V., Jr. (1969) in Fundamental Techniques in Virology (Habel, K. and Salzman, J.P., eds.) pp. 334—362, Academic Press, New York.
14 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
15 Potter, J.D. and Gergely, J. (1975) J. Biol. Chem. 250, 4628—4633
16 Martonosi, A. and Feretos, R. (1964) J. Biol. Chem. 239, 648—658
17 Ashby, C.D. and Walsh, D.A. (1972) J. Biol. Chem. 247, 6637—6642
18 Ashby, C.D. and Walsh, D.A. (1973) J. Biol. Chem. 248, 1255—1261
19 Lipmann, F. and Tuttle, L.C. (1945) J. Biol. Chem. 159, 21—28
20 Carstens, M. and Weller, M. (1979) Biochim. Biophys. Acta 551, 420—431
21 Kirchberger, M.A. and Tada, M. (1976) J. Biol. Chem. 254, 725—729
22 Caswell, A.H., Baker, S.P., Boyd, H., Potter, L.T. and Garcia, M. (1978) J. Biol. Chem. 253, 3049-3054
23 Brostrom, C.O., Hunkeler, F.L. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1961—1967
24 Horl, W.H., Jennissen, H.P. and Heilmeyer, L.M.G. (1978) Biochemistry 17, 759—766
25 Horl, W.H. and Heilmeyer, L.M.G. (1978) Biochemistry 17, 766—772
26 Wray, H.L. and Gray, R.R. (1977) Biochim. Biophys. Acta 461, 441—459
27 LaRaia, P.J. and Morkin, E. (1974) Circ. Res. 35, 298—306
28 Tada, M., Kirchberger, M.A. and Katz, A.M. (1975) J. Biol. Chem. 250, 2640—2647
29 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1—79
30 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94—111
31 Weber, A., Herz, R. and Reiss, I. (1966) Biochem. Z. 345, 329—369
32 Yamada, S., Yamamoto, T. and Tonomura, Y. (1970) J. Biochem. 67, 789—794
33 Johnson, E.M., Maeno, H. and Greengard, P. (1971) J. Biol. Chem. 247, 7731—7739
34 Dousa, T.P., Sands, H. and Hechter, O. (1972) Endocrinology 91, 757—763
35 Roses, A.D. and Appel, S.H. (1973) J. Biol. Chem. 248, 1408—1411
36 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1971) J. Biol. Chem. 247, 6135—6139