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A Calmodulin-dependent Protein Kinase System from Skeletal Muscle Sarcoplasmic Reticulum

PHOSPHORYLATION OF A 60,000-DALTON PROTEIN*

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The calmodulin plus Ca^{2+}-dependent phosphorylation of a 60,000-dalton protein was detectable in muscle homogenates and in purified sarcoplasmic reticulum membranes. Phosphorylation was enhanced when purified sarcoplasmic reticulum vesicles were extracted with 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid at pH 8.0 to remove endogenous calmodulin and lower the level of residual phosphorylation. Phosphorylation of the 60,000-dalton protein was stimulated by NaF, reached maximal levels of about 55 pmol/mg of protein within 90 s and was not affected by cAMP. Half-maximal stimulation of phosphorylation required 0.1 μM calmodulin and maximal phosphorylation required 0.6 μM calmodulin; in the presence of 0.6 μM calmodulin, maximal phosphorylation required 0.3 μM Ca^{2+}. Calmodulin-dependent phosphorylation of the 60,000-dalton protein was inhibited by trifluoperazine with a K_i of 5 μM. The pH optimum of phosphorylation was below 6.0 and was inhibited over 90% at pH 8.0.

The purified 60,000-dalton protein contained phosphoserine and phosphothreonine but no phosphotyrosine. Two-dimensional gel electrophoresis and immunoprecipitation showed that the 60,000-dalton phosphoprotein was not calsequestrin. Endo-β-*N*-acetylglucosaminidases H and D digestion failed to alter its molecular weight, indicating that it was not a glycoprotein.

A functional role for the phosphorylation system is suggested by the observations that ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid extraction, which removes endogenous calmodulin and lowers endogenous phosphorylation levels, and high pH, which inhibits phosphorylation, lead to greatly diminished Ca^{2+} accumulation by sarcoplasmic reticulum vesicles.

Calmodulin is an intracellular Ca^{2+} binding protein that activates a variety of enzymes in the presence of Ca^{2+} (1). It has been found to activate phosphodiesterase (2, 3), adenylate cyclase (4, 5), erythrocyte Ca^{2+} + Mg^{2+}-ATPase (6, 7), myosin light chain kinase (8), and phosphorylation of membranes from various tissues (9). Endogenous substrates for the calmodulin-dependent, membrane protein kinase differed in each tissue examined and were usually distinct from cAMP-dependent protein kinase substrates in the same tissues (9).

In canine cardiac sarcoplasmic reticulum, stimulation of Ca^{2+}-dependent ATPase and Ca^{2+} uptake has been reported to result from the phosphorylation of a 22,000-dalton membrane protein designated phospholamban (10). Phospholamban is phosphorylated by both the catalytic subunit of cAMP-dependent protein kinase (10) and by a calmodulin-dependent, membrane-bound protein kinase (11). Recent evidence suggests that it is phosphorylation by the calmodulin-dependent system which stimulates calcium uptake (11).

Skeletal muscle sarcoplasmic reticulum membranes have recently been shown to contain an endogenous protein phosphorylation system (12). Several phosphorylated proteins were observed when phosphorylation was carried out in the presence of 1 mM EGTA[1] but in the absence of cAMP and exogenous protein kinase. Phosphorylation was observed in proteins of M_r = 64,000, 42,000, and 20,000. The 64,000-dalton phosphoprotein was more concentrated in heavy sarcoplasmic reticulum vesicles and its phosphorylation was inhibited by dantrolene, an inhibitor of Ca^{2+} release. The effect of calmodulin on phosphorylation was not examined.

In this paper we describe the discovery of the calmodulin-dependent phosphorylation of a 60,000-dalton membrane protein which purifies with skeletal muscle sarcoplasmic reticulum membranes.

MATERIALS AND METHODS

Materials—Sodium dodecyl sulfate, acrylamide, *N,N'*-methylenebisacrylamide, 2-mercaptoethanol, and *N,N,N',N'*-tetramethylethylene-diamine were purchased from Bio-Rad. Protein A Sepharose 4B was obtained from Pharmacia. [^{32}P]phosphate and $^{45}Ca^{2+}$ were obtained from Amersham. [γ-^{32}P]ATP was either purchased from Amersham or synthesized from ^{32}P and ADP by photophosphorylation (13) and purified by the method of Glynn and Chappel (14). Calmodulin, prepared from bovine cerebral cortex by the method of Teo *et al.* (3), was a generous gift from Dr. Jerry Wang, University of Manitoba. Phosphoserine and phosphothreonine were obtained from Sigma. Phosphotyrosine, prepared by the method of Mitchell and Lunan (15), was a generous gift from Dr. Phillip E. Branton, McMaster University. Trifluoperazine was a gift from Smith, Kline, and French, Canada Ltd. Endo H[1] (*Streptomyces griseus*) was a product of Health Research Inc. Albany, NY. Endo D (*Diplococcus pneumoniae*) was a product of Miles.

Preparation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic

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[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TFP, trifluoperazine; Endo H and Endo D, endo-β-*N*-acetylglucosaminidases H and D.

reticulum vesicles were prepared from rabbit skeletal muscle according to the method of MacLennan (16) as modified by Campbell and MacLennan (17). Crude sarcoplasmic reticulum vesicles were obtained in the initial 44,000 × *g* centrifugation step (13). Mild trypsin digestion of sarcoplasmic reticulum vesicles was performed according to Stewart and MacLennan (18). Calsequestrin was purified according to MacLennan and Wong (19) except that the deoxycholate extract was first passed through a column of Bio-Gel A-5m to remove higher molecular weight aggregates of the ATPase and the 53,000-dalton glycoprotein. The 53,000-dalton glycoprotein was purified as described previously (17).

Sarcoplasmic reticulum vesicles were also prepared from rat skeletal muscle, chicken breast muscle, and porcine leg muscle by the same procedures (17). Cardiac sarcoplasmic reticulum was prepared from canine hearts as described in Ref. 20.

EGTA Washing of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles at 10 mg of protein/ml were incubated for 15 min at 0 °C in a solution of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM histidine, pH 8.0 (buffer A), containing 1 mM EGTA and then centrifuged for 15 min at 105,000 × *g* (21). Vesicles were resuspended twice in their own supernatant and the last time in fresh buffer A containing 1 mM EGTA. The protein was adjusted to 20 mg/ml. The EGTA extract was boiled for 5 min, centrifuged to remove insoluble material, dialyzed, and then assayed for its ability to stimulate phosphorylation of the 60,000-dalton protein.

Calmodulin-dependent Phosphorylation of Sarcoplasmic Reticulum Vesicles—Phosphorylation of sarcoplasmic reticulum vesicles was carried out at 30 °C in 100 μl of the kinase buffer described by Schulman and Greengard (9): 50 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 10 mM $MgCl_2$, 10 mM NaF, 0.1 mM dithiothreitol, 0.2 mM EGTA, 0.5 mM $CaCl_2$, 10 μM [γ-^{32}P]ATP (1000 cpm/pmol), and 20–100 μg of sarcoplasmic reticulum protein in the presence or absence of 0.6 μM calmodulin. After 30 s, the reaction was terminated by the addition of 50 μl of a solution containing 6% SDS, 188 mM Tris-HCl, pH 6.8, 3% 2-mercaptoethanol, 1 mM EGTA, 30% glycerol, 0.001% bromphenol blue. Samples were heated at 100 °C for 2 min and applied quantitatively to SDS-polyacrylamide slab gels. After electrophoretic separation, radioactive bands were localized by autoradiography and cut out of the dried gel, and the radioactivity was quantitated by liquid scintillation counting. Calmodulin-dependent phosphorylation is given as the difference between the phosphorylation in the presence and absence of calmodulin.

Gel Electrophoresis and Autoradiography—The analysis of phosphoproteins by SDS polyacrylamide slab gel electrophoresis was performed using the discontinuous buffer system of Laemmli (22) in 1.5-mm thick slab gels of 7.5% acrylamide. Phosphoproteins were also analyzed on Weber and Osborn (23) SDS-polyacrylamide slab gels. Two-dimensional gel electrophoresis was carried out as previously described (24). Autoradiography of dried slab gels was performed using Kodak X-Omat film and a Dupont Cronex Lightning Plus enhancing screen. Apparent M_r values were calculated from a graph of relative mobilities *versus* log M_r of standard proteins.

Phosphoamino Acid Analysis—EGTA-washed sarcoplasmic reticulum vesicles were phosphorylated with [γ-^{32}P]ATP in the presence of calmodulin and then subjected to preparative gel electrophoresis. The 60,000-dalton phosphoprotein was eluted from preparative Laemmli slab gels in a solution of 0.1% SDS and 1% NH_4HCO_3. SDS was removed by incubation and centrifugation in the cold and the phosphoprotein was precipitated with 20% trichloroacetic acid. The precipitated protein was dissolved in 6 N HCl and hydrolyzed for 2 h at 100°C. The HCl was evaporated and the hydrolysate was analyzed on cellulose thin layer plates by two-dimensional high voltage electrophoresis at pH 3.5 and 1.9 (25). The positions of the reference phosphoamino acids phosphoserine, phosphothreonine, and phosphotyrosine were determined by ninhydrin staining and the positions of ^{32}P-labeled amino acids were determined by autoradiography.

Immunoprecipitation—Immunoprecipitates were obtained using the appropriate antiserum and protein A Sepharose beads (26). EGTA-washed sarcoplasmic reticulum vesicles were phosphorylated with [γ-^{32}P]ATP in the presence of calmodulin as described above. Phosphorylation was stopped by the addition of 20 volumes of a solution of 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 100 Kallikrein inactivating units/ml aprotinin. Aliquots of 1 ml were incubated for 3 h at 4°C with constant stirring with 20 μl of the appropriate antiserum and 250 μl of protein A Sepharose beads suspended in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100 (1:10, v/v). Sepharose beads with bound antibody were collected by centrifugation and washed three times with 1 ml of a solution of 100 mM Tris-HCl, pH 7.0, 200 mM LiCl, 0.1% 2-mercaptoethanol and twice with 1 ml of a solution of 1 mM Tris-HCl, pH 7.0, 150 mM NaCl. The preparation of antisera against the ATPase, calsequestrin, and the high affinity calcium binding protein has been described previously (27). The antiserum against the 53,000-dalton glycoprotein was obtained exactly as described for the calsequestrin antibody (27) except that the protein injected into a sheep was the 53,000-dalton glycoprotein extracted from SDS-polyacrylamide gels.

Removal of Carbohydrate from Sarcoplasmic Reticulum Proteins—Oligosaccharide chains were removed from sarcoplasmic reticulum glycoproteins with Endo H or Endo D. Calmodulin-dependent or independent phosphorylation of EGTA-washed sarcoplasmic reticulum vesicles was performed as described above except that the phosphorylation reaction was stopped by the addition of 10% SDS so that the final protein-to-SDS ratio was 1:2 (w/w). The samples were heated at 100 °C for 1 min, cooled, and diluted with a solution of 50 mM sodium citrate, pH 5.5, to bring the SDS concentration to 0.2%. PMSF was added to 1 mM and Endo H to 50 milliunits/ml. After 6 h at 37 °C, the reaction was terminated by the addition of SDS sample buffer followed by incubation at 100 °C for 1 min. Endo D digestion was carried out in the same manner at pH 6.5. Control incubations were carried out in the absence of Endo H or Endo D.

Assays—Protein was determined by the method of Lowry *et al.* (28) using bovine serum albumin as a standard. Ca^{2+} uptake was measured using Millipore filtration (29). ATPase activity was measured as previously described (16).

RESULTS

Calmodulin-dependent Phosphorylation of Sarcoplasmic Reticulum Vesicles—An autoradiogram (*A*) illustrating the incorporation of ^{32}P from 10 μM [γ-^{32}P]ATP into sarcoplasmic reticulum proteins is shown in Fig. 1. In the absence of calmodulin or in the presence of calmodulin and absence of Ca^{2+} (Fig. 1*A*), no phosphorylation was observed. Ca^{2+} plus calmodulin stimulated the phosphorylation of three specific sarcoplasmic reticulum proteins. The molecular weight of the major phosphorylated protein was 60,000 while the molecular weights of the two minor phosphorylated proteins were 85,000 and 20,000 (Fig. 1*A*). The stained gel (Fig. 1*B*) shows the three major sarcoplasmic reticulum proteins: the Ca^{2+} + Mg^{2+} ATP-

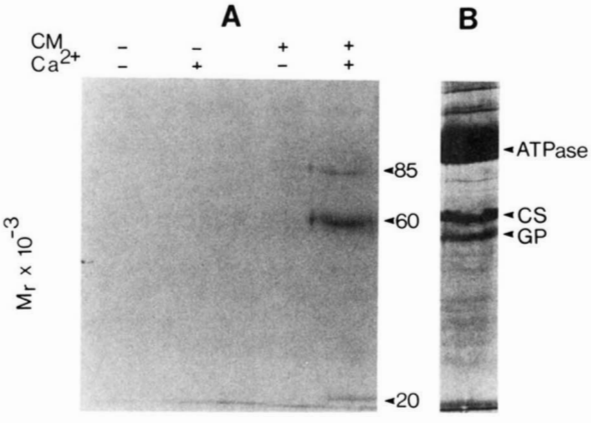

FIG. 1. **Calmodulin-dependent phosphorylation of sarcoplasmic reticulum vesicles.** Sarcoplasmic reticulum vesicles (100 μg) were preincubated in 100 μl of a solution containing 50 mM 1,4-piperazinediethanesulfonic acid, pH 7.0, 10.0 mM NaF, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and either 0.2 mM EGTA (minus calcium) or 0.2 mM EGTA plus 0.5 mM $CaCl_2$ (plus calcium) and in the presence (+) or absence (−) of 1.0 μg calmodulin (*CM*) (0.6 μM). Phosphorylation was initiated by the addition of 10 μM [γ-^{32}P]ATP (10^3 cpm/pmol) at 30 °C and stopped after 30 s with 50 μl of a SDS solubilizing solution (see "Materials and Methods"). After heating at 100 °C for 2 min, the samples were analyzed by SDS gel electrophoresis. *A*, autoradiograph illustrating calmodulin-dependent phosphorylation of three proteins of M_r = 20,000, 60,000, and 85,000 (*arrows*); *B*, stained gel lane illustrating the major sarcoplasmic reticulum proteins; ATPase, Ca^{2+} + Mg^{2+}-ATPase, 105,000 daltons; *CS*, calsequestrin, 63,000 daltons; and *GP*, 53,000-dalton glycoprotein.

ase, M_r = 105,000; calsequestrin, M_r = 63,000; and the intrinsic glycoprotein, M_r = 53,000. The 60,000-dalton phosphorylated protein ran at the front of the calsequestrin band and was distinguishable as a separate protein band when small amounts of protein were run on the gel.

Analyses of various fractions, obtained during the purification of sarcoplasmic reticulum vesicles from rabbit skeletal muscle, indicated that calmodulin-dependent phosphorylation of the 60,000- and 20,000-dalton substrates was concentrated with the sarcoplasmic reticulum membrane (Fig. 2). Phosphorylation of the 60,000-dalton protein was observed in the crude microsomal fraction (Fig. 2*B*) and in the purified sarcoplasmic reticulum (Fig. 2*C*) but was completely absent from the 44,000 × *g* supernatant (Fig. 2*A*). Phosphorylation of the 85,000-dalton protein was diminished upon purification of the sarcoplasmic reticulum from crude microsomes.

Since the sarcoplasmic reticulum was isolated in buffers which did not contain EDTA or EGTA, it was probable that endogenous calmodulin was still bound to the membranes. To test this possibility, we washed the vesicles with 1 mM EGTA at pH 8.0 to remove any endogenous calmodulin and then assayed for phosphorylation. Fig. 2*D* illustrates that the sarcoplasmic reticulum had enhanced calmodulin-dependent phosphorylation of the 60,000-dalton protein following EGTA washing. Quantitation of the phosphorylation observed in Fig. 2 is given in Table I. Calmodulin-dependent phosphorylation of the 60,000-dalton protein was doubled during purification of the sarcoplasmic reticulum from the microsomal fractions, and EGTA washing of the sarcoplasmic reticulum vesicles resulted in a further 3-fold enhancement of phosphorylation. Phosphorylation levels of 30–60 pmol/mg of sarcoplasmic reticulum protein were routinely observed in the 60,000-dalton band. Phosphorylation of the 20,000- and 85,000-dalton proteins was also enhanced after EGTA washing. Calmodulin-independent and Ca^{2+}-independent phosphorylation of a 53,000-dalton protein was not affected by the EGTA washing procedure (not shown). Incubation in the presence of EGTA also resulted in enhanced phosphorylation, although it was not as effective as EGTA washing. KCl-washed sarcoplasmic reticulum also had enhanced calmodulin-dependent phosphorylation of the 60,000-dalton protein but higher concentrations of calmodulin were required to obtain maximum phosphorylation.

The EGTA extract and the boiled EGTA extract of sarcoplasmic reticulum stimulated phosphorylation of the 60,000-dalton protein in EGTA-washed sarcoplasmic reticulum as shown in Fig. 3. With the assumption that kinase is activated

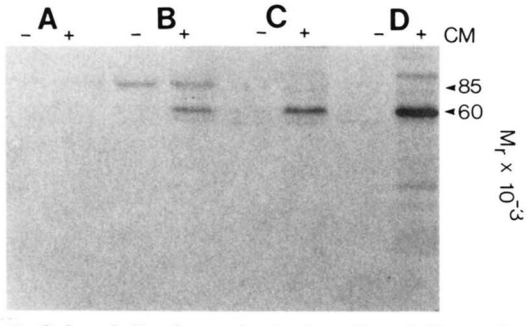

FIG. 2. **Calmodulin-dependent phosphorylation of various fractions from the purification of sarcoplasmic reticulum vesicles.** Phosphorylation was carried out as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$ and in the absence (−) or presence (+) of 0.6 μM calmodulin (*CM*) *A*, autoradiogram illustrating the calmodulin-dependent phosphorylation of the 60,000-dalton protein in the 44,000 × *g* supernatant; *B*, 44,000 × *g* microsomal fraction; *C*, sarcoplasmic reticulum vesicles; and *D*, EGTA-washed sarcoplasmic reticulum vesicles.

TABLE I

Quantitation of calmodulin-dependent phosphorylation of the 60,000-dalton protein

Phosphorylation was carried out as described under "Materials and Methods," in the presence or absence of 0.6 μM calmodulin (CM) for 30 s at 30 °C.

Fraction	^{32}P incorporation		
	−CM	+CM	Difference
	pmol/mg		
44,000 × *g* supernatant	0.6	1.1	0.5
44,000 × *g* microsomal fraction	1.2	7.5	6.3
Sarcoplasmic reticulum	1.9	13.4	11.5
EGTA-washed sarcoplasmic reticulum	2.4	37.5	35.1

only by calmodulin, this experiment demonstrates the presence of calmodulin in these extracts. The boiled EGTA supernatants had no endogenous phosphorylation activity. Since the optimal amount of extract needed to stimulate phosphorylation varied from preparation to preparation, we assume that the calmodulin content of sarcoplasmic reticulum was variable.

Mild tryptic digestion of sarcoplasmic reticulum vesicles, resulting in only a single cleavage of the ATPase (18), brought about the loss of calmodulin-dependent phosphorylation of the 60,000-dalton protein. Similarly, heating sarcoplasmic reticulum for 1 min at 60 °C resulted in the loss of phosphorylation activity. It is not known whether the kinase, the 60,000-dalton substrate, or both were destroyed by trypsin or heat.

We observed calmodulin-dependent phosphorylation of the 60,000-dalton protein in rat, chicken, and pig skeletal muscle sarcoplasmic reticulum but not in canine cardiac sarcoplasmic reticulum, where the major calmodulin-dependent phosphorylation occurred in a protein of 22,000 daltons, presumably phospholamban (10, 11).

Characterization of Calmodulin-dependent Phosphorylation—When the phosphorylation assay was carried out in the absence of 10 mM NaF, ^{32}P incorporation reached a peak after 30 s and then declined until it reached a constant level which was approximately half the maximal level of phosphorylation. This behavior suggested that an equilibrium was attained between phosphorylation and dephosphorylation. The addition of NaF to the reaction buffer doubled the phosphorylation level and inhibited dephosphorylation. This effect of NaF is consistent with its known inhibitory action on phosphoprotein phosphatase (30). The time course of calmodulin-dependent ^{32}P incorporation into the 60,000- and 20,000-dalton proteins from EGTA-washed vesicles in the presence of NaF is shown in Fig. 4. Phosphorylation of the 60,000-dalton protein was maximal at 55 pmol/mg after 90 s and remained constant over a 10-min period while the 20,000-dalton protein required between 5 and 10 min to reach its maximal phosphorylation of 30 pmol/mg. NaF did not completely inhibit dephosphorylation when incubations were carried out over 30 min.

A dose-response curve for calmodulin-dependent phosphorylation of the 60,000-dalton protein in EGTA-washed vesicles is shown in Fig. 5. When the reaction was terminated at 30 s, a time when phosphorylation was increasing linearly, the half-maximal rate of phosphorylation was reached with 0.1 μM calmodulin. Calmodulin dependence was similar for phosphorylation of the 20,000- and 60,000-dalton proteins. If we assume that the sarcoplasmic reticulum contains about 4 nmol of ATPase/mg of protein and that the ratio of ATPase to glycoprotein is about 2:1, then at 1.0 μM calmodulin and 1 mg of sarcoplasmic reticulum protein/ml, the molar ratio of calmodulin to ATPase was about 1:4 and of calmodulin to glycoprotein was about 1:2.

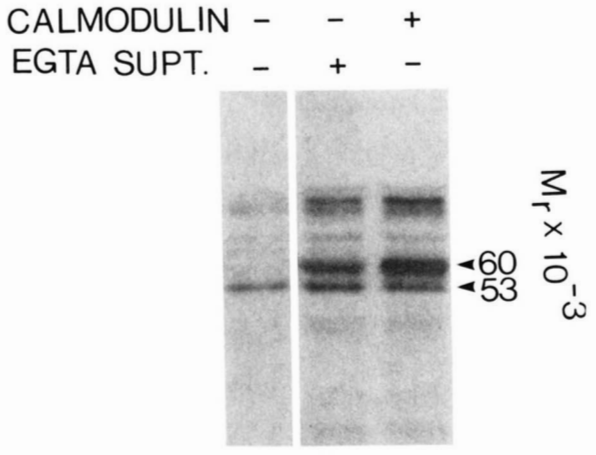

FIG. 3. **Effect of boiled extract on phosphorylation of EGTA-washed sarcoplasmic reticulum.** Sarcoplasmic reticulum vesicles were washed with 1 mM EGTA, pH 8.0, as described under "Materials and Methods." The EGTA extract was boiled for 5 min and centrifuged to remove insoluble material. Phosphorylation was carried out as described in Fig. 1 with 0.2 mM EGTA, 0.5 mM $CaCl_2$, in the presence of 30 μg of EGTA extract or 1.0 μg of calmodulin. The autoradiogram illustrates phosphorylation of the 60,000-dalton protein which is dependent on Ca^{2+} and either calmodulin or the EGTA extract and the Ca^{2+} and calmodulin-independent phosphorylation of the 53,000-dalton protein. *SUPT*, supernatant.

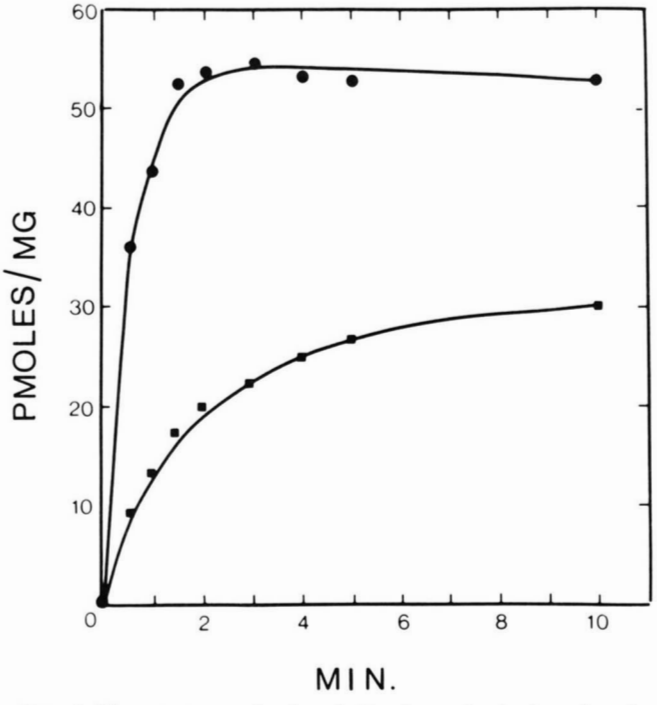

FIG. 4. **Time course of calmodulin-dependent phosphorylation of the 60,000- and 20,000-dalton proteins.** Phosphorylation of EGTA-washed sarcoplasmic reticulum was carried out as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$, 0.6 μM calmodulin and quantitated following autoradiography by liquid scintillation counting of the radioactive 60,000- (●) and 20,000- (■) dalton protein bands. Controls were run in the absence of calmodulin. Phosphorylation is given as picomoles of ^{32}P incorporated/mg of sarcoplasmic reticulum protein in a calmodulin-dependent reaction.

Calmodulin is extremely sensitive to Ca^{2+} concentration. The effects of various concentrations of Ca^{2+} on the phosphorylation of the 60,000-dalton protein is shown in Fig. 6. Half-maximal stimulation occurred with a free Ca^{2+} concentration of about 0.3 μM and the calcium binding sites interacted in a positively cooperative manner with a Hill coefficient of n = 2.5. This is consistent with previous observations (32, 33) that, under various conditions, half-maximal activation of calmodulin-dependent phosphodiesterase and half-maximal binding of Ca^{2+} to calmodulin occur in the range of 0.2 to 3.0 μM free Ca^{2+}.

When phosphorylation was carried out at various concentrations of [γ-^{32}P]ATP, maximal phosphorylation of the 60,000-dalton protein occurred with 50 μM ATP and several phosphorylated bands were observed representing the activities of both calmodulin-dependent and independent kinases. As the concentration of [γ-^{32}P]ATP was reduced, phosphorylation of the 60,000-dalton protein rather selectively remained

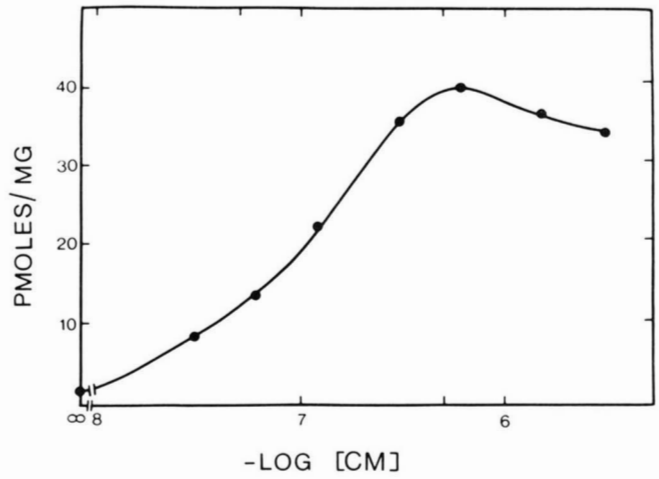

FIG. 5. **Calmodulin dependence of phosphorylation of the 60,000-dalton protein.** Phosphorylation of EGTA-washed sarcoplasmic reticulum was carried out as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$ with varying concentrations of calmodulin (*CM*). Quantitation was carried out by liquid scintillation counting of the radioactive 60,000-dalton protein band following localization by autoradiography. Controls were run in the absence of calcium. ^{32}P incorporated/mg of sarcoplasmic reticulum protein which is dependent on the presence of calcium is shown.

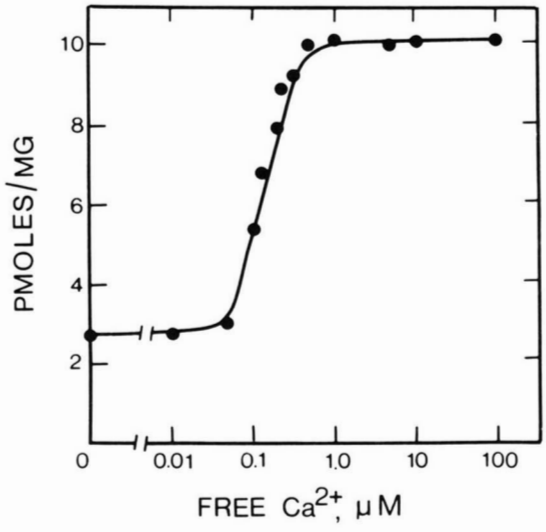

FIG. 6. **Calcium dependence of phosphorylation of the 60,000-dalton protein.** Phosphorylation of EGTA-washed sarcoplasmic reticulum was carried out as described in Fig. 1 in the presence of 0.3 mM EGTA, 0.6 μM calmodulin, and various amounts of calcium. Free Ca^{2+} concentrations were calculated using an apparent binding constant for Ca^{2+} EGTA of 7.61×10^6 M^{-1} (31). Quantitation was carried out by liquid scintillation counting of the radioactive 60,000-dalton protein following localization by autoradiography. Phosphorylation is given as total picomoles of ^{32}P incorporated/mg of sarcoplasmic reticulum protein.

high. These observations suggest that the calmodulin-dependent kinase had a very high affinity binding site for ATPase. The effect of cAMP on the calmodulin-dependent phosphorylation of the 60,000-dalton protein is shown in Fig. 7. cAMP in the range between 10^{-8} and 10^{-5} M had little influence on the calmodulin-dependent kinase system. The calmodulin-independent phosphorylation of a 53,000-dalton protein was greatly inhibited by cAMP (Fig. 7). We have previously shown (17) that a minor 53,000-dalton protein is adventitiously bound to sarcoplasmic reticulum and that this protein binds cAMP. This had led us to believe that the phosphorylated 53,000-dalton protein is the type II regulatory subunit of cAMP-dependent protein kinase. If this were true, then the addition of cAMP would dissociate the regulatory and catalytic subunits, leading to a diminished phosphorylation of the regulatory subunit, thereby accounting for our present observations.

The phosphoamino acids in the 60,000-dalton protein were identified by high voltage electrophoresis on thin layer plates following partial hydrolysis of the SDS-purified protein in 6 N HCl. Fig. 8 shows that the 60,000-dalton phosphoprotein contained [^{32}P]phosphoserine and [^{32}P]phosphothreonine. No [^{32}P]phosphotyrosine was observed even when overloaded samples were run. Treatment of the phosphoprotein with 1 N KOH at 55 °C for 2 h resulted in the loss of greater than 95% of the bound [^{32}P]phosphate. This treatment would destroy phosphothreonine but not phosphotyrosine, providing further evidence that there was no [^{32}P]phosphotyrosine present in the 60,000-dalton protein. Approximately equal amounts of radioactivity were observed in each phosphoamino acid when the phosphoserine and phosphothreonine spots were counted.

The 60,000-dalton Phosphoprotein Is Not Calsequestrin or a Glycoprotein—Since the 60,000-dalton phosphorylated protein had a mobility in Laemmli gels similar to calsequestrin (M_r = 63,000), we tested whether it might be calsequestrin using a two-dimensional gel electrophoresis system that we developed to separate calsequestrin from the rest of the sarcoplasmic reticulum proteins (24). Fig. 9 shows that the 60,000-dalton phosphorylated protein did not leave the diagonal as did calsequestrin, indicating that its apparent M_r was 60,000

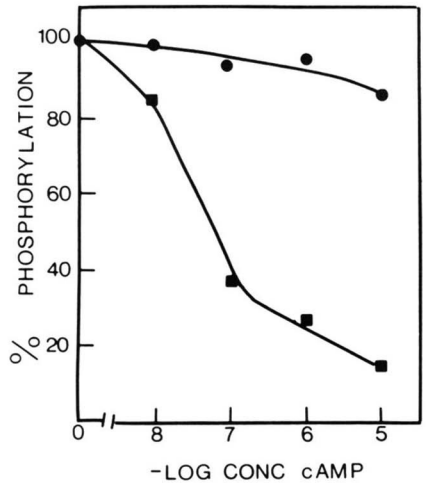

FIG. 7. **Effect of cAMP on the phosphorylation of the 60,000-dalton and 53,000-dalton protein.** Sarcoplasmic reticulum vesicles were phosphorylated as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$, 0.6 μM calmodulin, and various concentrations of cAMP (10^{-8}–10^{-5} M). The 60,000-dalton (●) and 53,000-dalton (■) phosphoproteins were localized by autoradiography and quantitated by liquid scintillation counting. Percentage of phosphorylation of the 60,000- and 53,000-dalton proteins in the absence of cAMP is plotted against increasing cAMP concentrations (*CONC*).

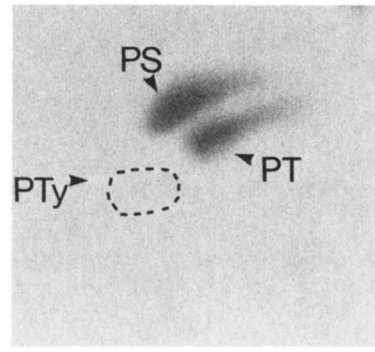

FIG. 8. **Phosphoamino acid analysis of the partially purified 60,000-dalton phosphoprotein.** Sarcoplasmic reticulum proteins were phosphorylated in the presence of calmodulin and subjected to preparative SDS gel electrophoresis. The 60,000-dalton phosphoprotein was localized by autoradiography, excised, and eluted. After precipitation with 20% trichloroacetic acid, the protein was subjected to acid hydrolysis in 6 N HCl at 110 °C for 2 h and the hydrolysate was separated by two-dimensional paper electrophoresis at pH 3.5 and 1.9 (25). The autoradiogram showed radioactivity in phosphoserine (*PS*) and phosphothreonine (*PT*) and not in phosphotyrosine (*PTy*).

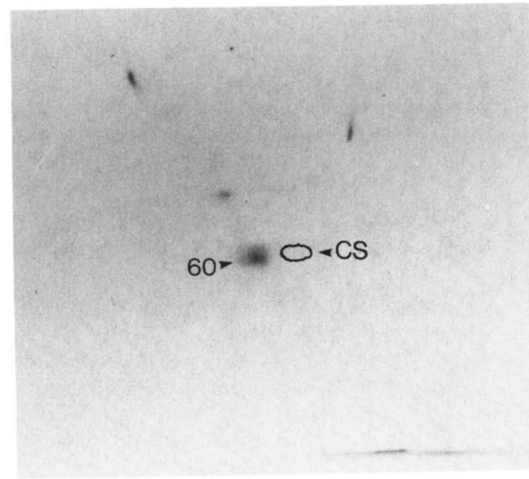

FIG. 9. **Two-dimensional gel electrophoresis of phosphorylated sarcoplasmic reticulum.** Sarcoplasmic reticulum vesicles were phosphorylated as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$, 0.6 μM calmodulin except that the solubilizing buffer of Weber and Osborn (23) was used to stop phosphorylation. The proteins were separated in the first dimension on a Weber and Osborn (23) slab gel (7.5% polyacrylamide) and in the second dimension on a Laemmli (22) slab gel (7.5% polyacrylamide) as described (24). The autoradiograph showing the 60,000-dalton phosphoprotein on the diagonal of the two-dimensional slab gel is shown. Calsequestrin (*CS*) stained by Coomassie blue is designated.

in both Laemmli and Weber and Osborn gel systems. It should be noted that, on overexposed autoradiograms, some ^{32}P was detected in the region of calsequestrin but it was not calmodulin-dependent.

We have also used immunoprecipitation to show that the 60,000-dalton phosphorylated protein was not calsequestrin (Fig. 10). The phosphorylation and dephosphorylation reactions were stopped by the addition of the immunoprecipitation buffer which contained 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, and immunoprecipitates were obtained within 2 h using antiserum and protein A Sepharose. Separation of ^{125}I-labeled calsequestrin from other ^{125}I-labeled sarcoplasmic reticulum proteins (Fig. 10*A*) by immunoprecipitation is shown in Fig. 10*B*. Control antiserum and antiserum against calsequestrin were not able to immunoprecipitate the ^{32}P-labeled 60,000-dalton protein (Fig. 10*D*). Antiserum against the ATP-

ase, the 53,000-dalton glycoprotein, or high affinity calcium binding protein were also ineffective. We also observed that purified calsequestrin, added to the calmodulin-dependent phosphorylation assay system, did not become phosphorylated.

Since the sarcoplasmic reticulum contains a minor glycoprotein of approximately 60,000 daltons, we have tested whether the ^{32}P-labeled 60,000-dalton protein is a glycoprotein using Endo H or Endo D. Endo H digestion of the sarcoplasmic reticulum proteins after phosphorylation did not alter the mobility of the 60,000-dalton phosphoprotein, indicating that it was not a glycoprotein (Fig. 11*A*). The protein of 53,000 daltons, which was phosphorylated in a calmodulin-independent reaction, was also not altered in mobility after Endo H digestion, suggesting that it was not a glycoprotein (Fig. 11*B*). Similar results were obtained with Endo D digestion (not shown). The calmodulin-independent phosphoprotein was a minor component of the 53,000-dalton band since the bulk of the 53,000-dalton band was reduced to 49,000 daltons with Endo H (Fig. 11*C*). We believe that the 53,000-dalton phosphoprotein is the type II regulatory subunit of the cAMP-dependent protein kinase.

Effect of pH on Phosphorylation of Sarcoplasmic Reticulum—The calmodulin-dependent phosphorylation of the 60,000-dalton protein was strongly pH-dependent, falling off sharply as pH was increased above 6.0. The effect of pH on phosphorylation of the 60,000-dalton protein in sarcoplasmic reticulum vesicles and in EGTA-washed sarcoplasmic reticulum vesicles is shown in Fig. 12. Phosphorylation in sarcoplasmic reticulum was slightly more sensitive to elevated pH than was phosphorylation in EGTA-washed vesicles. In order to rule out diminished phosphorylation because of higher ATPase activities, we measured ^{32}P liberation at various pH values and determined that [γ-^{32}P]ATP was not limiting in either phosphorylation reaction. Calmodulin-independent phosphorylation of the 53,000-dalton protein was insensitive to pH (not shown). Moreover, the detergent-solubilized phosphorylation system which was depleted of ATPase was also pH-dependent (not shown).

Effect of Trifluoperazine on Phosphorylation and on Ca^{2+} Accumulation by Sarcoplasmic Reticulum—Calmodulin-dependent phosphorylation of the 60,000-dalton protein was inhibited by trifluoperazine (TFP) (Fig. 13). Half-maximal inhibition was dependent on the sarcoplasmic reticulum protein concentration. In the presence of 0.6 μM calmodulin, 5, 25, and 70 μM TFP was required for half-maximal inhibition of phosphorylation at sarcoplasmic reticulum concentrations of 20, 100, and 1000 μg/ml, respectively. This may reflect a nonspecific partitioning of TFP into the sarcoplasmic reticulum membrane. Trifluoperazine had no effect on the calmodulin-independent phosphorylation of a 53,000-dalton protein.

Trifluoperazine was also found to be an effective inhibitor of calcium accumulation by sarcoplasmic reticulum. At a

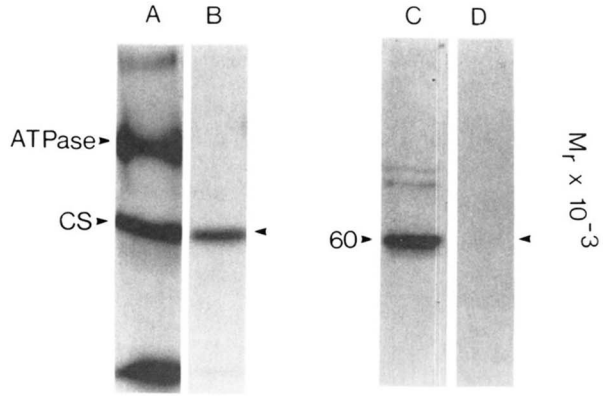

FIG. 10. **Immunoprecipitation of calsequestrin.** Sarcoplasmic reticulum vesicles were iodinated with ^{125}I using an immobilized lactoperoxidase-glucose oxidase system (*Lane A*). The immunoprecipitate of ^{125}I-calsequestrin (*CS*) (*Lane B*) was obtained with antiserum against purified calsequestrin. Calmodulin-dependent phosphorylation of sarcoplasmic reticulum showing the M_r = 60,000 phosphoprotein is illustrated in *Lane C*. *Lane D* illustrates that the phosphoprotein was not immunoprecipitated with antiserum against purified calsequestrin.

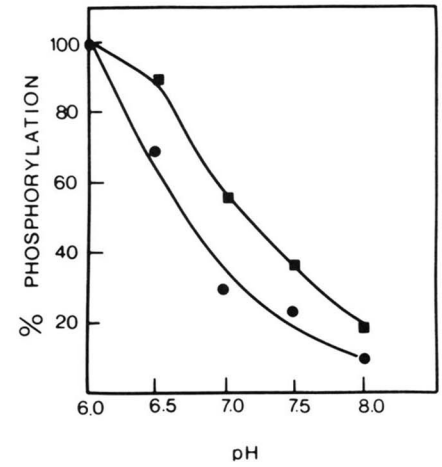

FIG. 12. **Effect of pH on the phosphorylation of the 60,000-dalton protein in different vesicles.** Sarcoplasmic reticulum (●) or EGTA-washed sarcoplasmic reticulum (■) were phosphorylated in the presence of calmodulin at various pH values according to Fig. 1. Percentage of phosphorylation of the 60,000-dalton protein as compared with the maximum phosphorylation at pH 6.0 is plotted against the pH of the phosphorylation buffer. Controls were run in the absence of calmodulin.

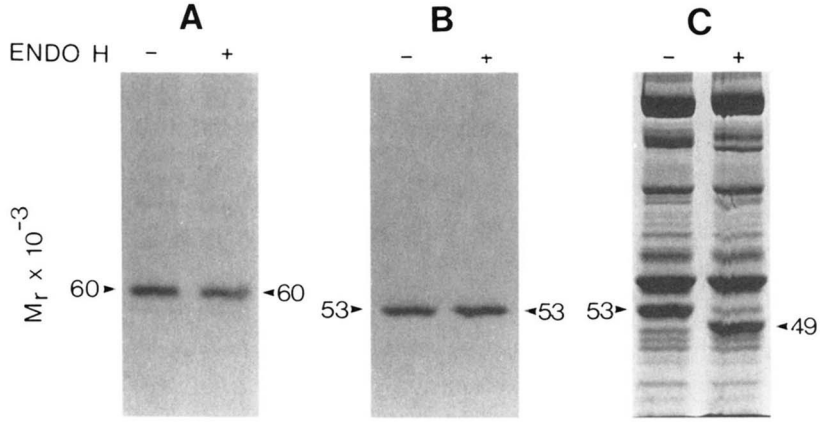

FIG. 11. **Endo H digestion of phosphorylated sarcoplasmic reticulum.** Calmodulin-dependent phosphorylation of sarcoplasmic reticulum and calmodulin-independent phosphorylation of sarcoplasmic reticulum were carried out as described in Fig. 3. Phosphorylation was stopped by SDS and boiling. Denatured proteins were then incubated in 50 mM Na-citrate, pH 5.5, and 1 mM PMSF at 37 °C for 6 h in the absence (−) or presence (+) of Endo H. Autoradiographs show the absence of a mass change in the 60,000-dalton phosphoprotein (*A*) or the 53,000-dalton phosphoprotein (*B*), respectively. The stained gel (*C*) shows the mass change (53,000 to 49,000) in the 53,000-dalton glycoprotein.

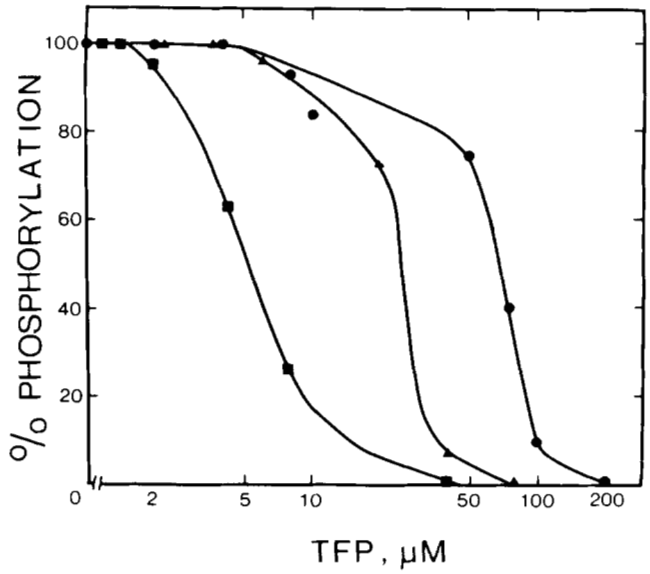

FIG. 13. **Trifluoperazine inhibition of calmodulin-dependent phosphorylation of the 60,000-dalton protein.** Phosphorylation of the EGTA-washed sarcoplasmic reticulum was carried out as described in Fig. 1 in the presence of 0.2 mM EGTA, 0.5 mM $CaCl_2$, 0.6 μM calmodulin, 20 (■), 100 (▲), or 1000 μg/ml (●) sarcoplasmic reticulum and various concentrations of trifluoperazine. Controls were run in the absence of calmodulin. Quantitation was carried out by liquid scintillation counting of the radioactive 60,000-dalton protein band following localization by autoradiography. Percentage of phosphorylation of the 60,000-dalton protein as compared with maximum phosphorylation in the absence of TFP is plotted against increasing TFP concentrations in the phosphorylation buffer.

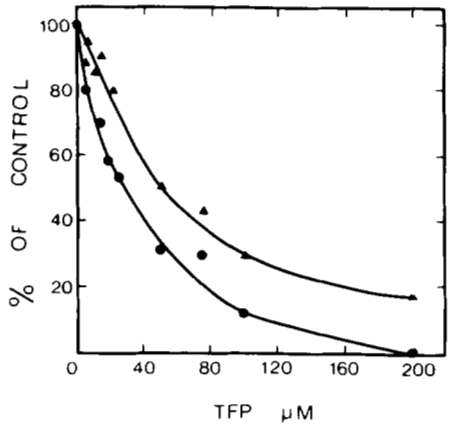

FIG. 14. **Trifluoperazine inhibition of Ca^{2+} uptake and ATPase of sarcoplasmic reticulum vesicles.** Ca^{2+} uptake and Ca^{2+} + Mg^{2+}-ATPase activities were measured at 20 °C in the presence of various concentrations of trifluoperazine in 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 20 mM histidine, pH 6.8, 5 mM oxalate, 5 mM ATP at a protein concentration of 20 μg/ml. Ca^{2+} uptake was measured with ^{45}Ca using Millipore filtration, and Ca^{2+} + Mg^{2+}-ATPase activity was measured with [γ-^{32}P]ATP. Percentage of activity, rate of Ca^{2+} uptake (●), or rate of Ca^{2+} + Mg^{2+}-ATPase (▲) are plotted against increasing TFP concentrations.

sarcoplasmic reticulum protein concentration of 20 μg/ml, half-maximal inhibition of calcium accumulation required approximately 30 μM trifluoperazine (Fig. 14). At concentrations up to 100 μM, trifluoperazine did not have any significant effect on release of Ca^{2+} from preloaded vesicles, suggesting that it did not inhibit Ca^{2+} accumulation through nonspecific membrane damage leading to Ca^{2+} release. The Ca^{2+} + Mg^{2+}-ATPase activity was inhibited by TFP (Fig. 14) but at slightly higher concentrations of TFP than were required for inhibition of Ca^{2+} uptake. Thus, the Ca^{2+}/ATP ratio fell as TFP concentrations were increased in the reaction mixture.

DISCUSSION

In the present study, we have found that an endogenous, calmodulin-dependent kinase in skeletal muscle sarcoplasmic reticulum specifically catalyzes the phosphorylation of 20,000- and 60,000-dalton proteins. Phosphorylation rates differed for the two proteins. In addition, the 20,000- and 60,000-dalton phosphoproteins differed in their physical properties; the 60,000-dalton protein was more readily detergent-soluble than the 20,000-dalton protein.

The calmodulin-dependent phosphorylation system has several components. The substrates are 20,000- and 60,000-dalton proteins. We do not know whether one or the other of these is also a kinase which can be autophosphorylated. We have noted that a 53,000-dalton glycoprotein of the sarcoplasmic reticulum binds 8-N_2-ATP with high affinity, suggesting that it might possess protein kinase activity (42). As yet, we have not been able to fractionate the system to the point where we can decide which protein is the kinase.

Calmodulin is clearly a component of the system. We have shown that an endogenous activator of phosphorylation can be extracted from sarcoplasmic reticulum by EGTA-washing at pH 8.0. We believe that this represents at least a remnant of calmodulin normally associated with the sarcoplasmic reticulum membrane.

A phosphatase activity is also found in sarcoplasmic reticulum. Varsanyi and Heilmeyer (34) have studied this activity previously and have shown that it is inhibited by 10 μM Ca^{2+} and activated in the presence of EGTA. We have noted that dephosphorylation of the 60,000-dalton protein occurs even in the presence of Ca^{2+} but that the activity is diminished by the addition of NaF, a known inhibitor of protein phosphatase (30). We have also noted that levels of phosphorylation of the 60,000-dalton protein are enhanced after vesicles have been either extracted with EGTA or merely incubated with EGTA. This would be expected if phosphorylation were inhibited through the Ca^{2+}-calmodulin complex and if phosphatase activity were stimulated, thereby decreasing the basal level of phosphorylation.

The pH dependence of the calmodulin-dependent phosphorylation system seems to be a function of the protein kinase rather than of the phosphatase since the experiments reported here were performed in the presence of NaF. Moreover, when membranes were phosphorylated at pH 6.0 for 2 min and then the pH was raised to 8.0, the level of phosphorylation remained unchanged, indicating that the phosphatase activity was not enhanced by alkaline pH.

There have been several reports that calsequestrin is a kinase (35, 36) and that it is phosphorylated (12, 35, 36). In our investigation, we have shown that the calmodulin-dependent kinase in sarcoplasmic reticulum does not phosphorylate calsequestrin. Although we did observe some ^{32}P associated with calsequestrin on two-dimensional gels, this phosphorylation did not require calmodulin.

Calmodulin is known to exist in skeletal muscle largely as an intermyofibrillar protein. Harper *et al.* (37) have localized calmodulin in skeletal muscle with peroxidase-labeled antiserum and fluorescein-labeled antiserum. The entire I band, including the Z-line was shown to stain for calmodulin but only slight staining was observed in the A band. Staining in the I band was greatly reduced after α-amylase digestion while the intermyofibrillar staining remained. These results suggest that the intermyofibrillar staining for calmodulin may be due to calmodulin bound to the sarcoplasmic reticulum. Calmodulin has not been identified in SDS-polyacrylamide gels of

isolated sarcoplasmic reticulum. It has been reported that a minor component of the sarcoplasmic reticulum appears as an intensely fluorescent band on SDS gels of cycloheptaamylose-fluorescein complex-labeled sarcoplasmic reticulum and this band also shows a high degree of labeling by [^{35}S]sulfanilic acid diazonium salt (38). These results indicate that a minor component of approximately 20,000 daltons is bound to the external face of the sarcoplasmic reticulum. This might be membrane-bound calmodulin.

In considering the physiological role of the phosphorylation system, it is relevant to note that the system seems to be integral to the sacroplasmic reticulum membrane and not an adventitious component of another metabolic system. If phosphorylation of the 60,000-dalton protein plays a regulatory role, then it must be in regulation of Ca^{2+} uptake, storage, or release, the known functions of the sarcoplasmic reticulum. We do not think that it is relevant to Ca^{2+} uptake. The stoichiometry between the ATPase, which transports Ca^{2+}, and the phosphorylated 60,000-dalton protein is about 60:1. This is in contrast to the stoichiometry of 1:1 between the ATPase of cardiac sarcoplasmic reticulum and its regulator, phospholambam (10). Moreover, the 60,000-dalton protein, unlike phospholamban, is not tightly associated with the ATPase. Moreover, the purified ATPase, devoid of the phosphorylation system, transports Ca^{2+} after reconstitution into high phospholipid vesicles (39). Similarly, we have no reason to assume that the phosphorylated protein is involved in Ca^{2+} storage, a role that has been assigned to calsequestrin (19).

A strong circumstantial case can, however, be developed for a role of the phosphorylation system in Ca^{2+} release as in the regulation of Ca^{2+} release. The phosphorylation system would be inactive in a relaxed muscle since the sarcoplasmic reticulum would lower cytoplasmic Ca^{2+} below the threshold for calmodulin activation of the kinase. Thus, phosphorylation would be observed only subsequent to Ca^{2+} release. In another communication (40), we have described experiments which lead us to believe that the Ca^{2+} release channel in sarcoplasmic reticulum is controlled, in part, by a proton gradient generated during Ca^{2+} uptake and in part by Ca^{2+} released by the sarcoplasmic reticulum. The addition of proton ionophores or slightly alkaline medium to Ca^{2+}-loaded sarcoplasmic reticulum in skinned muscle fibers led to an instant twitch response, suggesting that the Ca^{2+} release channels opened as soon as the pH gradient was dissipated. The fact that a twitch rather than a prolonged contraction was observed suggested that the Ca^{2+} release channel closed very rapidly following the first burst of Ca^{2+} release. It is difficult to visualize that this could happen through a rapid regeneration of the proton gradient by Ca^{2+} re-uptake, especially since the Ca^{2+} channel is probably also open to proton movements. If the channnel were doubly regulated, however, it could be closed by a Ca^{2+}-dependent reaction such as the phosphorylation that we have observed.

In order to consider the significance of these observations further, we propose that the Ca^{2+} release channel is open in the absence of a proton gradient across the sarcoplasmic reticulum membrane and when the 60,000-dalton protein is dephosphorylated. As Ca^{2+} flows out, it binds to calmodulin, stimulating phosphorylation of the 60,000-dalton protein which closes a gate in the channel. As Ca^{2+} is transported inward, a proton gradient is generated which may close another gate in the channel. When external Ca^{2+} is lowered below the threshold of binding to calmodulin, the 60,000-dalton protein becomes dephosphorylated by an endogenous phosphatase and the gate opens. However, the proton-sensitive gate is still closed. The membrane is now poised for release stimulated by dissipation of the proton gradient.

Experiments that suggest that phosphoryıation may be effective in controlling Ca^{2+} release are the following. The pH optimum for ATP hydrolysis by Ca^{2+}-ATPase is 7.5 whereas the pH optimum for Ca^{2+} accumulation is below 7.0. Therefore, Ca^{2+}-to-ATP ratios fall off rapidly above pH 7.0 as Ca^{2+} accumulation becomes uncoupled from ATP hydrolysis. The pH optimum for phosphorylation of the 60,000-dalton protein was shown in this study to be below 7.0 and phosphorylation fell off strongly above pH 7.0 with similar kinetics as those observed for Ca^{2+} accumulation. If one assumes that the same partial reactions and conformational changes occur in the ATPase at pH 6.8 or 7.5, then Ca^{2+} should be transported inward at pH 7.5. Lack of Ca^{2+} accumulation could readily be accounted for, however, if the Ca^{2+} release channel were open at pH 7.5 so that Ca^{2+} pumped inward were released rather than accumulated. Since Ca^{2+} uptake is initiated by ATP or Ca^{2+} addition under experimental conditions, a proton gradient could not be formed unless the Ca^{2+} channel were closed by phosphorylation. The action of trifluoperazine to bring about a decreased Ca^{2+}/ATP ratio might also be a reflection of its ability to inhibit calmodulin-dependent phosphorylation, thereby enhancing Ca^{2+} release.

In experiments reported in 1972 (21), we noted that EGTA extraction of sarcoplasmic reticulum led to inhibition of Ca^{2+} accumulation by sarcoplasmic reticulum without an inhibition of ATP hydrolysis. In this paper, we have shown that these extracts contain calmodulin. Calcium accumulation could be restored in extracted particles by reconstitution with a heat-stable supernatant factor (21). The reconstitution required ATP, Ca^{2+}, and Mg^{2+}, conditions which would favor restoration of the calmodulin-dependent phosphorylation of the sarcoplasmic reticulum. Diamond *et al.* (41) have reported that incubation of sarcoplasmic reticulum with EGTA at 37 °C and neutral pH leads to enhanced rates of Ca^{2+} efflux without increasing the inulin-accessible space. We have observed that phosphorylation sites increase and Ca^{2+} accumulation decreases upon EGTA incubation at 37 °C and neutral pH. These observations can all be interpreted as indicating that EGTA extraction removes calmodulin and calcium, resulting in the dephosphorylation of the 60,000-dalton protein, increased Ca^{2+} release, and diminished Ca^{2+} accumulation. Experiments to provide further evidence for the proposed role for phosphorylation in Ca^{2+} release are currently underway.

We do not know whether the 60,000-dalton protein could itself be the Ca^{2+} release channel or whether it is also a kinase which would then function in a cascade affecting Ca^{2+} release. We believe that its concentration in the membrane would be sufficient to permit Ca^{2+} release since only a few open channels would be required to account for the rate of Ca^{2+} release observed *in situ*.

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