Biochimica et Biophysica Acta, 426 (1976) 57–72

BBA 77185

THE RATE OF CALCIUM UPTAKE INTO SARCOPLASMIC RETICULUM OF CARDIAC MUSCLE AND SKELETAL MUSCLE

EFFECTS OF CYCLIC AMP-DEPENDENT PROTEIN KINASE AND PHOSPHORYLASE *b* KINASE

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(Received June 17th, 1975)
(Revised manuscript received September 30th, 1975)

SUMMARY

Calcium transport into sarcoplasmic reticulum fragments isolated from dog cardiac and mixed skeletal muscle (quadriceps) and from mixed fast (tibialis), pure fast (caudofemoralis) and pure slow (soleus) skeletal muscles from the cat was studied. Cyclic AMP-dependent protein kinase and phosphorylase *b* kinase stimulated the rate of calcium transport although some variability was observed. A specific protein kinase inhibitor prevented the effect of protein kinase but not of phosphorylase b kinase. The addition of cyclic AMP to the sarcoplasmic reticulum preparations in the absence of protein kinase had only a slight stimulatory effect despite the presence of endogenous protein kinase.

Cyclic AMP-dependent protein kinase catalyzed the phosphorylation of several components present in the sarcoplasmic reticulum fragments; a 19000 to 21000 dalton peak was phosphorylated with high specific activity in sarcoplasmic reticulum preparations isolated from heart and from slow skeletal muscle, but not from fast skeletal muscle. Phosphorylase *b* kinase phosphorylated a peak of molecular weight 95000 in all of the preparations. Cyclic AMP-dependent protein kinase-stimulated phosphorylation was optimum at pH 6.8; phosphorylase *b* kinase phosphorylation had a biphasic curve in cardiac and slow skeletal muscle with optima at pH 6.8 and 8.0. The addition of exogenous phosphorylase *b* kinase or protein kinase increased the endogenous level of phosphorylation 25–100 %.

All sarcoplasmic reticulum preparations contained varying amounts of adenylate cyclase, phosphorylase *b* and *a* (*b*:*a* = 30:1), "debrancher" enzyme and glycogen

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Preliminary reports on this subject were presented at the Annual FASEB Meeting, Atlantic City, N.J., 1974; the Cyclic AMP Symposium, Vancouver, British Columbia, Canada, 1974; and combined meeting of the Biophysical Society and the American Society of Biological Chemists, Minneapolis, Minn, 1974.

(0.3 mg/mg protein), as well as varying amounts of protein kinase and phosphorylase *b* kinase which were responsible for a significant endogenous phosphorylation.

Thus, the two phosphorylating enzymes stimulated calcium uptake in the sarcoplasmic reticulum of a variety of muscles possessing different physiologic characteristics and different responses to drugs. In addition, the phosphorylation catalyzed by these enzymes occurred at two different protein moieties which makes physiologic interpretation of the role of phosphorylation difficult. While the role of phosphorylation in these mechanisms is complex, the presence of a glycogenolytic enzyme system may be an important link in this phenomenon. The sarcoplasmic reticulum represents a new substrate for phosphorylase *b* kinase.

INTRODUCTION

Calcium is a key ion in the modulation of excitation-contraction coupling in both cardiac and skeletal muscle [1]. The sarcoplasmic reticulum is thought to play a vital role because it is the principal calcium-sequestering membrane system responsible for muscle relaxation, and in skeletal muscle it also functions as the major site of "activator" calcium [2, 3]. In cardiac muscle, the internal store of calcium (activator calcium) necessary for cardiac contraction may be associated with the sarcoplasmic reticulum, the sarcolemma, or both. Because of these factors, the kinetics of calcium accumulation by sarcoplasmic reticulum have been extensively studied.

In view of the modulating influence of the sarcoplasmic reticulum on muscle function, the effect of various pharmacologic interventions known to induce positive inotropic effects has been investigated on isolated sarcoplasmic reticulum [4]. Entman et al. [5] and later Katz et al. [6] demonstrated the possible presence of an intracellularly located (probably sarcoplasmic reticulum) adenylate cyclase system in cardiac muscle similar to the one originally described for skeletal muscle by Rabinowitz et al. [7]. Protein kinases [8, 9] are relatively "nonspecific" enzymes in that they catalyze the transfer of the terminal phosphate of nucleoside triphosphates to serine or threonine present in various protein substrates including phosphorylase kinase kinase and a component of troponin [10–12].

Phosphorylase *b* kinase is a much more specific enzyme than protein kinase. To date, four substrates have been identified: phosphorylase *b*, troponin, casein and the enzyme itself (autophosphorylation) [8, 13, 14]. The physiological significance of cyclic AMP-protein kinase or phosphorylase b kinase-catalyzed phosphorylation of troponin is still unknown, since a specific function for phospho-troponin has not been found.

Because of the recently demonstrated association of protein kinase with cyclic AMP-mediated events, Kirchberger et al. [15] re-examined the problem of intracellular calcium control by sarcoplasmic reticulum in the heart as influenced by cyclic AMP and protein kinase. Using a procedure for isolation of sarcoplasmic reticulum developed in this laboratory, these authors reported that cyclic AMP stimulated a protein kinase-dependent phosphorylation of cardiac sarcoplasmic reticulum fragments, and this was associated with a specific stimulation of calcium transport. Kirchberger et al. [15] found no significant effect of cyclic AMP alone

and no significant endogenous protein kinase activity. These observations were also reported by LaRaia et al. [16, 17], who, in addition, found a significant amount of endogenous protein kinase; cyclic AMP in the absence of added protein kinase stimulated calcium uptake.

Phosphorylase *b* kinase from skeletal and cardiac muscles is very sensitive to calcium [18, 19]. It has been suggested by Ozawa [18] and by Krebs et al. [19] that this enzyme may play a general role in calcium-mediated muscle contraction, metabolism and regulation of the action of epinephrine. In the present communication, we have examined the postulate that intracellular calcium activity in cardiac muscle might be controlled by phosphorylation. Fast, slow, and mixed skeletal muscle preparations were compared to cardiac muscle preparations to test muscle-type specificity since Katz et al. [15, 20, 21] have suggested that skeletal muscle sarcoplasmic reticulum is insensitive to cyclic AMP and protein kinase.

Our results indicate that the two phosphorylating enzymes significantly stimulate the transport of calcium into sarcoplasmic reticulum vesicles from all muscle types regardless of the in vivo pharmacological sensitivity. Protein kinase and phosphorylase *b* kinase phosphorylate different proteins in sarcoplasmic reticulum, and the variation in the presence or quantity of these phosphorylated proteins is dependent on muscle species.

METHODS

I. *Preparation of sarcoplasmic reticulum fragments*

Sarcoplasmic reticulum fragments were prepared from canine heart and skeletal muscle by methods previously described in our laboratory, using a bicarbonate/azide buffer and homogenizing with a Polytron PT35 homogenizer [22]. Sarcoplasmic reticulum fragments from cat soleus, tibialis anterior, and caudofemoralis muscles were isolated by a modified procedure as follows: Cats of either sex were stunned by cervical dislocation and the hearts were excised. In the cold room, the muscles were cut into small pieces, minced, and homogenized twice (for 5 s each) in four volumes of bicarbonate/azide, pH 6.8, with a Polytron PT20 at a rheostat setting of 80 (about 3000 rev/min). The sarcoplasmic reticulum was isolated by differential centrifugation, according to the modification described in ref. 22.

II. *Assay of calcium uptake*

The assay of calcium uptake was similar to that described previously [23, 24] using a dual-beam spectrophotometer and the calcium-sensitive dye, murexide (ammonium purpurate). The final reaction mixture in 3.0 ml consisted of 40 mM Tris/maleate, pH 6.8, 0.1 M potassium chloride, 10 mM magnesium chloride, 60 μM calcium chloride, and 5 mM Tris or sodium oxalate. Murexide was present in concentrations of 0.1 to 0.2 mM and sarcoplasmic reticulum fragments were used in concentrations of 50 to 200 μg/ml of protein. Preincubation was started by the addition of 3.3 mM adenosine 5′-triphosphate (ATP) and either 50 μg of protein kinase in the presence and absence of cyclic AMP or 30 μg of phosphorylase *b* kinase at 30 °C. Calcium uptake reaction was initiated after 5–10 min of preincubation by the addition of 60 μm calcium which then resulted in a linear uptake rate. The usual

Millipore filtration technique was also employed using, in some experiments, concentrations of calcium below 1 μM, maintained by a suitable ethylene-bis (β-amino ethyl ether) *N,N'*-tetraacetic acid (EGTA) buffer as recommended by Katz et al. [1, 3, 7]. Calcium "binding" (i.e., in the absence of oxalate) could not be measured under the conditions of this experiment because this reaction occurs very rapidly and is significantly limited by the preincubation step with ATP. The latter is required for demonstration of the kinase-stimulated calcium accumulation.

III. *Assay of phosphorylation*

The phosphorylation reaction with protein kinase was measured in a volume of 0.4 ml at pH 6.5 in the presence of 50 mM phosphate buffer, 10 mM magnesium chloride, about 200 μg of sarcoplasmic reticulum protein, 0.3 mM EGTA, 10 mM potassium fluoride, 50 μg of protein kinase (Sigma), 2.5 mM theophylline and cyclic AMP as indicated. 20 μl of 4 mM [γ^{32}P]ATP was added to start the reaction after 10 min of preincubation at 30 °C. The incubation was carried out at 30 °C for 10 min. The reaction was terminated by addition of 3 ml of 12.5 % ice-cold trichloroacetic acid containing 1 mM ATP and 10 mM sodium phosphate. 0.2 ml of 0.6 % bovine serum albumin was added as a carrier, and the suspension was centrifuged for 10 min. The resultant pellet was solubilized in 1.0 M sodium hydroxide and precipitated in trichloroacetic acid. The pellet was washed three times, solubilized in 0.2 M NaOH, 10 ml of Beckman Fluoralloy TLA®-Bio-Solv®-Toluene Fluid was added, and radioactivity was measured in a liquid scintillation counter.

The phosphorylase *b* kinase assay was generally carried out at its optimum pH of 8.5 but was also done at lower pHs at which calcium uptake was usually measured (see above). The conditions were similar to those for the protein kinase assay with the exception of the presence of 10 mM β-mercaptoethanol and 50 mM Tris or Tris/maleate buffer and 30 μg of phosphorylase b kinase instead of protein kinase, and the exclusion of EGTA, theophylline and cyclic AMP.

To determine the specificity of both exogenous and endogenous phosphorylation, some reactions were carried out in duplicate in the presence or absence of 5 mM EGTA to remove calcium, which is required for muscle phosphorylase *b* kinase activity [18, 19].

IV. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The reactions were terminated by immediate cooling and three-fold dilution with 20 mM sodium phosphate buffer, pH 7.0, containing 5 mM ATP. The sarcoplasmic reticulum fragments were collected by centrifugation at 100000 $\times g$ for 30 min. The pellets were suspended in Na_2HPO_4 buffer containing 5 mM ATP, recentrifuged, and then solubilized with sodium dodecyl sulfate in a solution containing 1 % β-mercaptoethanol, 10 mM sodium phosphate, pH 7.1, 5 % sodium dodecyl sulfate (at least 5 mg/mg protein) and 5 % glycerin. The samples were applied to 5 or 7.5 % polyacrylamide gels containing 0.1 % sodium dodecyl sulfate with bromphenol blue as a reference marker. The samples were initially electrophoresed toward the anode for 10–14 min at a current of 2 mA/gel until the samples had penetrated the gels and then at 6 mA/gel for approximately 2.5 h with 0.1 % sodium dodecyl sulfate and 50 mM sodium phosphate, pH 7.1, as electrolytes. The gels were removed from their tubes by injecting water from a fine needle, fixed for 24 h in 10 % trichlo-

roacetic acid and scanned at 280 nm for protein. Gels were also frozen, sliced into 2 mm sections, and counted in a scintillation counter. The molecular weights of the observed protein and radioactive peaks were determined by utilizing appropriate markers and constructing a relative mobility curve; standards used were phosphorylase *a* (94000), bovine serum albumin (68000), ovalbumin (43000), pepsin (35000), chymotrypsinogen (25000) and cytochrome *c* (11700). Molecular weights listed in this manuscript were calculated from this curve.

Phosphorylase *b* kinase (Sigma) was activated using a modification of the trypsin activation method of Bröstrom et al. [27]. The enzyme was incubated at 30 °C, pH 6.8, for 5 min in concentrations of 2 mg/ml with 10 μg/mg of trypsin. The reaction was stopped after 5 min by addition of a ten-fold excess of soybean trypsin inhibitor. Control inactive enzyme was treated the same way except that the inhibitor was present from the beginning. Phosphorylase activity was measured by a modification of the method of Helmrich and Cori [28], which quantitates glucose 1-phosphate formation using phosphoglucomutase, glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate (NADP). "Debrancher" enzyme activity was measured by the method of Nelson et al. [29] and glycogen by the phenol sulfuric acid method [30].

V. General comments

All experiments were repeated at least three times, and the results varied very little both qualitatively and quantitatively. Because of the need for preincubation in order to observe enzyme-induced augmentation of calcium accumulation, it was not possible to analyze calcium binding in the absence of oxalate. Therefore, the results reported here describe effects on the linear calcium transport reaction effected by the presence of oxalate which continuously precipitates transported calcium. In some cases, data are shown using sarcoplasmic reticulum from a single tissue; these are, unless otherwise stated, representative of all preparations studied.

VI. Sources of chemicals and enzymes

Protein kinase and phosphorylase *b* kinase were obtained from Sigma and contained minimal contamination with other enzymes. Heat-stable protein kinase inhibitor was supplied by Drs. J. Corbin and R. Park of Vanderbilt University, to whom we are most grateful. In some experiments, we used partially purified preparations of protein kinase and phosphorylase *b* kinase kindly supplied by Dr. James T. Stull, University of California, San Diego and by Drs. Joe Beavo, Jr. and E. G. Krebs, University of California, Davis.

RESULTS

Calcium uptake

A significant augmentation of calcium transport in sarcoplasmic reticulum preparations from slow and fast skeletal muscle by protein kinase plus cyclic AMP was found (Table I). The stimulation effect required preincubation of the enzyme plus cyclic AMP with the sarcoplasmic reticulum preparation (data not shown); minimal stimulation was obtained with protein kinase alone or cyclic AMP (Table I). The effects of cyclic AMP were concentration-dependent (Fig. 1) in all tissues, with a

TABLE I

THE EFFECTS OF CYCLIC AMP AND PROTEIN KINASE ON CALCIUM UPTAKE BY SARCOPLASMIC RETICULUM FRAGMENTS

Reactions were carried out at 30 °C in 3 ml volume containing: 10 mM $MgCl_2$, 0.1 M KCl, 3.3 mM ATP, 60 μM $CaCl_2$, 5 mM sodium or Tris/oxalate, 40 mM Tris/maleate (pH 6.8) and sarcoplasmic reticulum fragments, 100-300 μg/ml. The preincubation was started with ATP and, at 10 min, the calcium uptake reaction initiated by the addition of calcium. Protein kinase, 15 μg/ml; cyclic AMP, $5 \cdot 10^{-6}$ M; and protein kinase inhibitor, 20 μl (sufficient to block phosphorylation, data not shown). In this and following tables and figures cardiac muscle was derived from dog and tibialis and soleus from cat.

Addition	Calcium uptake (nmol/mg/min)		
	Cardiac	Tibialis	Soleus
Control with preincubation	118.7	642.2	332.3
Control without preincubation	125.8	706.6	346.2
Cyclic AMP	122.2	679.9	380.8
Cyclic AMP+protein kinase	191.6	1000.0	432.6
Cyclic AMP+protein kinase +protein kinase inhibitor	125.1	637.8	350.0
Protein kinase	138.3	705.2	385.5

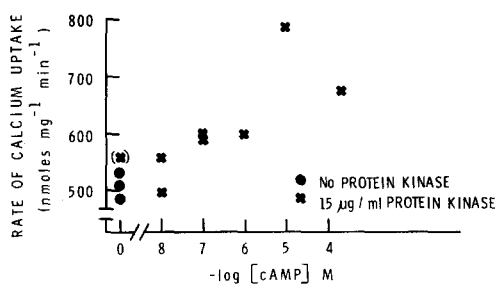

Fig. 1. Effect of cyclic AMP on protein kinase-mediated stimulation of the rate of calcium uptake in dog skeletal muscle (mixed) sarcoplasmic reticulum. Reaction as in Table I.

TABLE II

EFFECT OF PHOSPHORYLASE *b* KINASE ON CALCIUM UPTAKE BY SARCOPLASMIC RETICULUM FRAGMENTS

Reaction and preincubation conditions as in Table I, except phosphorylase *b* kinase was present and protein kinase and cyclic AMP were omitted.

Muscle	Calcium uptake (nmol/mg/min)	
	Control	Phosphorylase *b* kinase (10 μg/ml)
Cardiac	118.7	175.3
Tibialis	642.2	819.9
Soleus	166.1	265.2

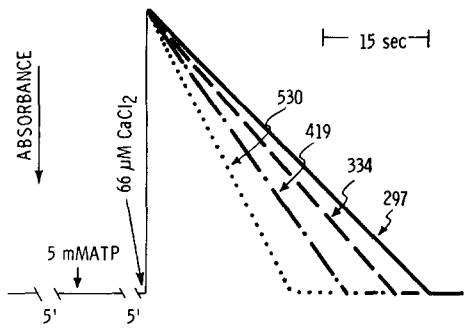

Fig. 2. Dual-wavelength traces of calcium uptake by cardiac relaxing system: Rates are expressed as nmoles calcium accumulated/ mg cardiac sarcoplasmic reticulum protein/min and are illustrated by numbers and arrows above the traces. Reaction conditions: 40 mM Tris/maleate (pH 6.8), 10 mM KCl, 10 mM $MgCl_2$, 5 mM sodium oxalate, 0.2 mM murexide and 50 μg cardiac sarcoplasmic reticulum protein/ml; when present, 50 μg protein kinase, 10^{-6} M cyclic AMP, 30 μg phosphorylase *b* kinase. Cuvette was equilibrated at 30 °C for 5 min, then 3.3 mM disodium ATP was added to the reaction. After 5 min of incubation, 60 μM calcium was added to initiate the uptake reaction. — = control; - - = cyclic AMP; ●— = phosphorylase *b* kinase; . . . . = protein kinase and cyclic AMP.

minimal effect at a concentration of 10^{-7} M. Cyclic guanosine monophosphate (GMP) had no effect (data not shown). The effect of cyclic AMP and protein kinase was inhibited by the heat-stable protein kinase inhibitor (Table I). Sarcoplasmic reticulum isolated from cat caudofemoralis, a pure fast muscle type, was affected by both protein kinase and phosphorylase b kinase in a manner similar to the other muscle types (data not shown).

Phosphorylase *b* kinase also markedly stimulated calcium uptake in the sarcoplasmic reticulum preparations from cardiac and in a variety of skeletal muscle preparations (Table II). It should be noted that this latter reaction was insensitive to cyclic AMP and protein kinase inhibitor; there was, therefore, no evidence of protein kinase contamination of the phosphorylase *b* kinase. The effects of phosphorylase b kinase and protein kinase on the rate of calcium uptake were evaluated by means of a dual-beam spectrophotometer, a sample trace of which is presented in Fig. 2.

These data were statistically significant ($P < .01$) but stimulation of calcium uptake was not invariant. In some preparations no stimulation was observed; however, if stimulation was found with addition of one phosphorylating enzyme (e. g. phosphorylase *b* kinase), the other (e.g., protein kinase) invariably stimulated calcium uptake. The non-stimulated sarcoplasmic reticulum preparations were characterized by control calcium uptake rates which approached the augmented rates in stimulated preparations containing high levels of endogenous phosphorylation. Control experiments done in the absence of sarcoplasmic reticulum protein or ATP did not show any calcium uptake.

Phosphorylation experiments

Protein kinase catalyzed the phosphorylation of all preparations tested (Table IIIA) and the reaction was stimulated by cyclic AMP in the presence or absence of exogenous protein kinase. The presence of endogenous cyclic AMP-

TABLE IIIA

PHOSPHORYLATION OF SARCOPLASMIC RETICULUM FROM VARIOUS MUSCLES BY EXOGENOUS PROTEIN KINASE

Phosphorylation reaction was at 30 °C in a volume of 0.4 ml in the presence of 50 mM sodium phosphate buffer (pH 6.5), 0.3 mM EGTA, 10 mM $MgCl_2$, 10 mM potassium fluoride, 0.2 mM [γ-^{32}P]-ATP, 10^{-6} M 3′,5′-cyclic AMP, 2.5 mM theophylline, 50 μg protein kinase and 200 μg sarcoplasmic reticulum protein for 10 min.

Muscle type	Phosphate Incorporation (pmoles/mg/10 minutes)			
	Control	cAMP	Protein kinase	Protein kinase+cAMP
Cardiac	405	525	768	1325
Soleus	510	750	821	1010
Tibialis	160	155	275	500

TABLE IIIB

PHOSPHORYLATION OF SARCOPLASMIC RETICULUM FROM VARIOUS MUSCLES BY ENDOGENOUS AND EXOGENOUS PHOSPHORYLASE *b* KINASE

Phosphorylation was carried out at 30 °C for 10 min in a volume of 0.4 ml in the presence of 50 M Tris buffer, pH 8.5, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.2 mM [γ-^{32}P]ATP, 30 μg of phosphorylase *b* kinase (Sigma), 200 μg of sarcoplasmic reticulum. The EGTA-insensitive phosphorylation has been subtracted.

Muscle Type	Number of Experiments	Phosphate incorporation (pmol/mg/10 min)	
		Sarcoplasmic reticulum	Sarcoplasmic reticulum+*b* kinase
Cat tibialis	5	1194±193 (S.D.)	1452±251
Cat soleus	5	333±135	621±226
Dog skeletal	5	484±121	659±249
Dog cardiac	8	237± 57	329±118

stimulated phosphorylation was demonstrable predominantly in cardiac and soleus sarcoplasmic reticulum; it was very low or absent in the tibialis preparation. The cyclic AMP-stimulated phosphorylation was completely inhibited by protein kinase inhibitor as previously described in the Methods section. Sarcoplasmic reticulum preparations from all muscles examined were phosphorylated by phosphorylase *b* kinase (Table IIIB). It was noted that sarcoplasmic reticulum fragments from tibialis exhibited a significant autophosphorylation catalyzed by phosphorylase *b* kinase (Table IIIB). In contrast to the phosphorylation catalyzed by phosphorylase *b* kinase, heat denaturation (78 °C for 10 min) only mildly attenuated phosphorylation by protein kinase (Table IV).

The phosphorylation of cardiac sarcoplasmic reticulum catalyzed by exogenous phosphorylase *b* kinase showed a broad pH optimum with distinct peaks at pH 6.8 and pH 8 (Fig. 3). Soleus sarcoplasmic reticulum showed a similar pH curve, but tibialis sarcoplasmic reticulum exhibited a very low (< 0.1) pH 6.8:8.5 ratio (data not shown). A similar pH pattern was found for endogenous phosphorylase *b* kinase-catalyzed phosphorylation in sarcoplasmic reticulum from cardiac and

TABLE IV

THE EFFECT OF HEAT DENATURATION ON PHOSPHORYLATION OF CARDIAC SARCOPLASMIC RETICULUM

Reaction mixture of A and B is the same as in Table IIIA and B. In phosphorylase *b* kinase (PbK) reactions, (A) 10 mM KF and 10 mM β-mercaptoethanol were added; a 50 mM Tris buffer was used in reactions at pH 8.5. Heat inactivation of sarcoplasmic reticulum (SR) was effected by incubation at 78 °C for 10 min.

Addition		pH	Phosphate incorporation (pmol/mg)	
			Control	Heat treated
A	SR	6.8	304	30.3
	SR	8.5	285	25.6
	SR	6.8	517	34.2
	SR+PbK	6.5	420	64.8
B	SR	6.5	480	2.1
	SR+PK	6.5	610	265
	SR+PK+cAMP	6.5	1720	904

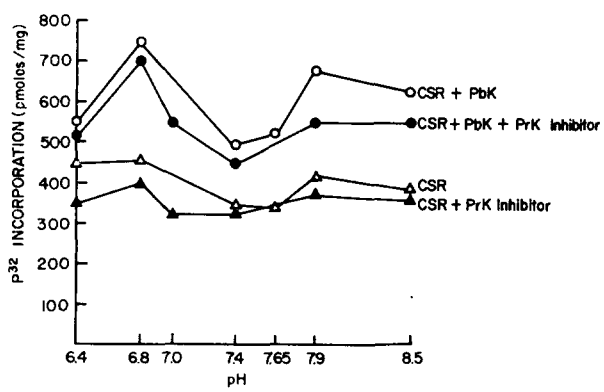

Fig. 3. Effect of pH on phosphate incorporation into cardiac sarcoplasmic reticulum. Reaction as in Tables III and IV. At lower pH, either 50 mM sodium phosphate or 40 mM Tris/maleate was substituted for 50 mM Tris. CSR, cardiac sarcoplasmic reticulum; PbK, phosphorylase *b* kinase; PrK, protein kinase.

soleus muscles. The possibility existed that endogenous protein kinase was: (1) distorting the total phosphorylation that could be attributed to phosphorylase *b* kinase, or (2) activating both the endogenous and exogenous phosphorylase *b* kinase so that the pH 6.8:8.5 approached 1.0, as described by Krebs and co-workers [19]. The experiments were repeated in the presence of protein kinase inhibitor in concentrations sufficient to inhibit virtually all cyclic AMP-stimulated phosphorylation. The presence of protein kinase inhibitor did not significantly alter the characteristics of the pH curve (Fig. 3). These data suggested the possibility that two endogenous substrates in sarcoplasmic reticulum with different pH specificities were phosphorylated by phosphorylase *b* kinase. Both exogenous and endogenous phosphorylase *b* kinase were able to phosphorylate phosphorylase *b* at pH 8.5 but were unable

TABLE V

SUBSTRATE AND pH DEPENDENCE OF ENDOGENOUS AND EXOGENOUS PHOSPHORYLASE *b* KINASE

Reactions as in Tables III and IV. Phosphorylase *b*, when present, 25 μg/ml; when phosphorylase *b* is the sole substrate, the units of phosphorylation are pmol/25 μg, and when both phosphorylase *b* and sarcoplasmic reticulum were present, the units are pmol/mg sarcoplasmic reticulum protein.

Additions			Phosphate incorporation (pmol/mg)	
Cardiac sarcoplasmic reticulum	Phosphorylase *b*	Phosphorylase *b* kinase	pH 6.8	pH 8.5
+	–	–	625	550
–	+	–	50	35
+	+	–	620	605
–	+	+	45	250
–	–	+	726	765
+	+	+	785	940

to phosphorylate significantly at pH 6.8 (Table V). The possibility exists, therefore, that the substrate for phosphorylation at pH 8.5 is phosphorylase *b* and the substrate at pH 6.8 may be something else. Maximal phosphorylase specific activity in cardiac sarcoplasmic reticulum is between 2–2.5 % that of purified phosphorylase (data not shown). Utilizing this estimate, each mg of sarcoplasmic reticulum contains 20–25 μg of phosphorylase which, if phosphorylated to the same degree as exogenous phosphorylase (10 pmol/μg exogenous phosphorylase *b* at pH 8.5; Table V), would account for 200-250 pmol/mg sarcoplasmic reticulum. This figure is only 30–40 % of the observed phosphorylation in Table V even at pH pH 8.5 and further suggests the presence of another substrate. It should be recalled that stimulation of calcium

TABLE VI

EFFECT OF CYCLIC AMP AND PROTEIN KINASE INHIBITOR ON FLUORIDE AUGMENTATION OF PHOSPHORYLASE *b* KINASE ACTIVITY: CARDIAC SARCOPLASMIC RETICULUM

Reaction as in Table IIIB except Tris/maleate 50 mM (pH 6.8) was used. PKI, heat stable protein kinase inhibitor; cyclic AMP, 10^{-5} M

Addition	Phosphate incorporation (pmol/mg/10 min)	
	Sarcoplasmic reticulum	Sarcoplasmic reticulum + phosphorylase *b* kinase
+KF Control	353	583
+cyclic AMP	557	801
+cyclic AMP+PKI	371	476
+PKI	411	442
−KF Control	107	218
+cyclic AMP	169	267

uptake was measured at pH 6.8. At any pH, heat inactivation or aging of a preparation markedly attenuated phosphorylase *b* kinase phosphorylation (in contrast to protein kinase phosphorylation) (Table IV).

The presence of adenylate cyclase and protein kinase in the preparation further complicated the observations regarding the pH dependency of phosphorylase *b* kinase activity. Since fluoride addition was necessary to inhibit endogenous phosphatase activity, the possibility existed that fluoride (by stimulation of cyclic AMP formation → protein kinase activation of phosphorylase *b* kinase) might augment the phosphorylation at pH 6.8 through activation of phosphorylase *b* kinase. To test this hypothesis, the experiments were performed in the presence of 10^{-5} M cyclic AMP (Table VI). The results suggested that the fluoride effect was primarily on phosphatase activity rather than on activation of inactive phosphorylase *b* kinase.

Gel electrophoresis

A typical pattern for sarcoplasmic reticulum for soleus, tibialis and cardiac muscle revealed numerous protein peaks, the most prominent of which was at a molecular weight of approx. 95 000 (Figs. 4, 5, 6). It is possible that this peak represents phosphorylase *a* and/or calcium ATPase. In the presence of protein kinase and cyclic AMP, the majority of phosphorylation occurred in a 20 000 dalton component in both cardiac and soleus preparations (Figs. 4 and 6). Phosphorylase *b* kinase at pH 6.8 or 8.5 did not augment incorporation of ^{32}P into the 20 000 molecular weight protein but rather into a 95 000 component in sarcoplasmic reticulum preparations from all muscle types. In sarcoplasmic reticulum isolated from tibialis, both protein kinase and phosphorylase *b* kinase resulted in phosphate incorporation predominantly into the 95 000 dalton protein peak and no 20 000 dalton protein was demonstrable (Fig. 5). Incorporation of ^{32}P into this 95 000 dalton protein

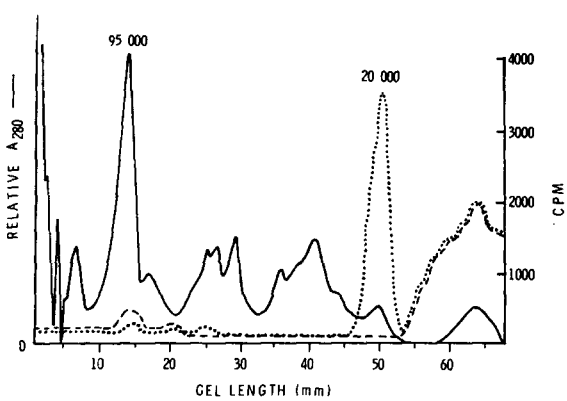

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sarcoplasmic reticulum isolated from cat soleus. Phosphorylation reactions as in Table III. The reaction was stopped by centrifugation at 100000 × *g* for 30 min. The pellets were treated as described in Methods section. The solubilized protein was applied to a 7.5 % sodium dodecyl sulfate-polyacrylamide gel and electrophoresed toward the anode as described in the text. —, absorbance at 280 nm; ● ● ●, phosphorylation in the presence of protein kinase; and - - - phosphorylation in the presence of phosphorylase *b* kinase.

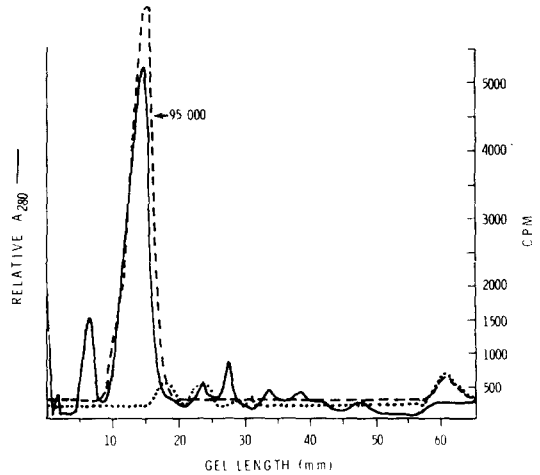

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of sarcoplasmic reticulum isolated from cat tibialis. Reaction and symbols as in Fig. 4.

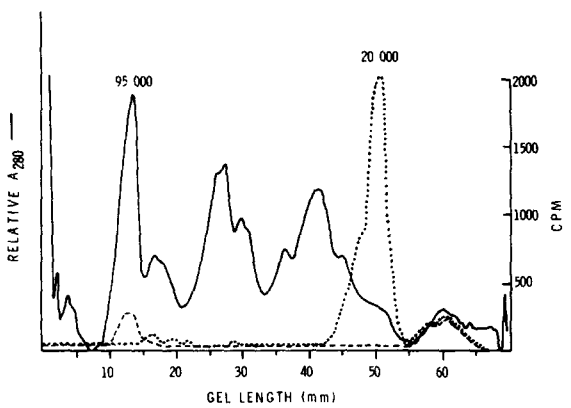

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cardiac sarcoplasmic reticulum. Reactions and symbols as in Fig. 4, except that protein kinase inhibitor was added to the phosphorylase *b* kinase incubation medium.

was inhibited only by EGTA (see Fig. 5, protein kinase conditions include EGTA) and not by protein kinase inhibitor. This suggests that activation of endogenous phosphorylase *b* kinase was responsible for the protein kinase effect in tibialis sarcoplasmic reticulum. In experiments in which fluoride was added, a smaller 20 000 dalton peak, presumably due to endogenous protein kinase, was phosphorylated in cardiac and soleus sarcoplasmic reticulum only. This peak was inhibited by protein kinase inhibitor.

As previously discussed, the sarcoplasmic reticulum preparations from all muscles had considerable endogenous phosphorylation so that addition of exogenous calcium-dependent or -independent protein kinase augmented endogenous

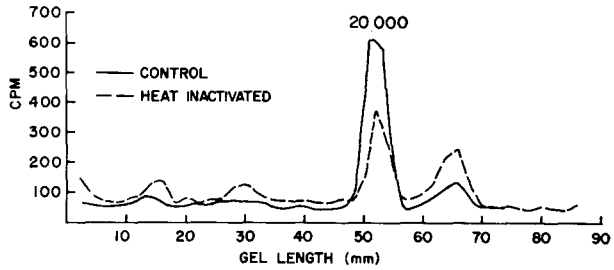

Fig. 7. Effect of heat inactivation on protein kinase-catalyzed phosphorylation of cardiac sarcoplasmic reticulum. Protein was incubated at 78 °C for 10 min and compared to a control incubated at 37 °C for the same period. After incubation, both preparations were cooled and then phosphorylated as described in Table III. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as in Figs. 4–6.

phosphorylation by varying amounts. High endogenous phosphorylation can occur in the absence of exogenous enzyme. In those cases, exogenous enzymes did not stimulate calcium uptake.

Other enzymes present

Phosphorylase ($b:a = 30.1$) with activities of 11.5 μmol/mg/min and "debrancher" enzyme, 50 nmol/mg/min, were found. Large quantities of glycogen were found in this preparation (approx. 0.3 mg/mg protein). Adenylate cyclase was also present in these preparations in ranges similar to those already reported [5–7], as previously mentioned. Nonspecific phosphatase activity was found predominantly in cardiac muscle and required the addition of fluoride to accurately assess phosphorylation. Mitochondrial contamination was minimal; there was no demonstrable $(Na^+ + K^+)$-ATPase [22], and we found no evidence of the presence of any myofibrillar components.

DISCUSSION

The finding of Katz and co-workers [15, 20, 21, 31, 32] that cyclic AMP and protein kinase augments calcium transport in cardiac sarcoplasmic reticulum and that this effect correlates with a specific phosphorylation of a 20000 molecular weight component named phospholamban suggested that a specific cyclic AMP-mediated event results in a change in calcium transport only in cardiac muscle. LaRaia and Morkin [17] found similar results except they reported the presence of endogenous protein kinase that was responsive to added cyclic AMP. Wray et al. [33] observed a cyclic AMP-stimulation of $[^{32}P]$ phosphate incorporation in cardiac microsomes. While we confirmed the data on calcium stimulation and on phosphorylation in cardiac and, in addition, in slow skeletal muscle, the results reported in this communication suggested that these effects are far more complex and of a more general nature. It should be noted that the augmented calcium uptake was observed not only by the murexide procedure, which requires relatively high calcium, but also by the traditional Millipore method, using conditions identical to those described by Katz et al. [6].

The following new observations have been made: (1) In the dog, both cardiac and mixed skeletal muscle sarcoplasmic reticulum fractions are stimulated with respect to calcium transport by protein kinase and cyclic AMP; in cat, both pure slow (soleus) and predominantly fast (tibialis) skeletal muscle sarcoplasmic reticulum are also affected in the same way. Recent experiments using a nearly pure fast skeletal muscle (caudofemoralis) have yielded the same results (data not shown). (2) Phosphorylase *b* kinase, which has heretofore been found to be a more specific enzyme than protein kinase, also stimulates calcium transport in various sarcoplasmic reticulum preparations. (3) The sarcoplasmic reticulum preparations described contain varying amounts of phosphorylase *b* kinase and protein kinase, as well as other enzymes linked to the glycogenolytic pathway. (4) Although both phosphorylase *b* kinase and protein kinase catalyze phosphorylation of proteins in the sarcoplasmic reticulum preparations, only protein kinase phosphorylates a 20000 molecular weight component of cardiac muscle sarcoplasmic reticulum described by Katz and co-workers [20, 21, 31, 32]. However, this component was also phosphorylated in cat soleus (slow, 34) but not in cat tibialis (fast, 34). Katz and his colleagues [15] have postulated that cardiac muscle or possible slow skeletal muscle responding to catecholamines with a decreased relaxation time [35, 36] do so because of a specific effect of cyclic AMP-dependent protein kinase which leads to phosphorylation of a specific protein component of molecular weight of 20000; this in turn causes an increase in calcium uptake. They have demostrated this in cardiac muscle only. We have confirmed the observation in cardiac muscle but also find the same stimulation of calcium uptake occurring in skeletal muscle regardless of the in vivo rate of shortening (from pure slow to pure fast fiber types). The fast skeletal muscle demonstrates this phenomenon in the absence of phosphorylation of a 20 000 dalton protein. Thus, there is no evidence to support the contention that the speed of shortening of a muscle influences either phosphorylation of a specific protein or calcium uptake [15]. These observations strongly suggest a more complex relationship with respect to pharmacologic or physiologic actions.

The phosphorylation of a 95000 molecular weight component by phosphorylase *b* kinase suggested that perhaps calcium ATPase was phosphorylated. However, since phosphorylase (94 000 molecular weight) is present in large amounts and since phosphorylase *b* kinase is most effective in phosphorylating this component, some phosphorylation found at this level is likely due to the presence of phosphorylase *b*. We have duplicated this finding using a purified phosphorylase *b* kinase kindly supplied by Dr. J.T. Stull. Gross and Mayer [37] suggest that our data (reported in preliminary form at Cyclic AMP Symposium, Vancouver, British Columbia, 1974) are consistent with phosphorylation of phosphorylase, "a likely contaminant of sarcoplasmic reticulum protein...". The unusual pH spectrum of phosphorylation in cardiac or soleus sarcoplasmic reticulum by both exogenous and endogenous phosphorylase *b* kinase suggests, however, that there may be another substrate or, alternatively, that membrane-associated phosphorylase *b* may be altered, in contrast to a purified enzyme. To further complicate this interpretation, it should be noted that autophosphorylation of phosphorylase *b* kinase can occur [19], although this reaction is considered to be very slow at pH 6.8, so that the likelihood of autoactivation of the enzyme is quite small. The altered pH characteristics of phosphorylase *b* kinase when phosphorylating other substrates is similar to the phenomenon de-

cribed by Stull et al. [13] for phosphorylation of a component of skeletal troponin by phosphorylase *b* kinase.

We have shown for the first time that phosphorylase *b* kinase catalyzes phosphorylation of muscle sarcoplasmic reticulum and stimulates calcium uptake in a manner quite similar to that reported for protein kinase. It was thought that this enzyme was highly specific, catalyzing primarily phosphorylation of phosphorylase *b*, although recently, phosphorylation of troponin has been reported [13].

From these data, it is difficult to relate phosphorylation of a specific sarcoplasmic reticulum protein component of 20 000 molecular weight or any other protein to the calcium transport activity of these membrane fragments. An at ractive possibility is that glycogenolysis may affect calcium uptake activity in response to some alteration in the microenvironment of the cell. Meyer et al. [38] have indicated that the enzymes for glycogenolysis and the sarcoplasmic reticulum in skeletal muscle may be intimately associated and that the glycogenolytic enzymes function in a highly specific way distinct from the behavior of the isolated enzymes. The present data suggest that membrane systems which modulate intracellular calcium for contraction and relaxation may be related to muscle metabolism. Catecholamines, which augment contraction and accelerate relaxation rate, may act by increasing cyclic AMP and calcium intracellularly. Calcium would activate phosphorylase b kinase which subsequently stimulates glycogenolysis and calcium uptake into the sarcoplasmic reticulum.

ACKNOWLEDGEMENTS

The authors extend their appreciation to Mr. Thomas Futch for his expert technical assistance. This work was supported by USPHS Grant HL 07906, HL 17269 (National Center for Research and Demonstration), HL 13870, HL 05925, Contract NIH 71-2493, and American Heart Association, Houston Chapter, Texas Affiliate. Dr. Mark L. Entman is an Investigator of the Howard Hughes Medical Institute. Dr. Lois K. Lane was a postdoctoral fellow of USPHS HL 02102.

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