

Phosphorylation of Heavy Sarcoplasmic Reticulum Vesicles: Identification and Characterization of Three Phosphorylated Proteins

Kevin P. Campbell* and Adil E. Shamoo**

Department of Radiation Biology and Biophysics, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642

Summary. Heavy sarcoplasmic reticulum vesicles derived from the terminal cisternae of the sarcoplasmic reticulum have been shown to contain endogenous protein kinase activity and associated substrate proteins. Heavy vesicles were phosphorylated at room temperature in 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.4) and 10 μM γ-³²P-ATP. ³²P-phosphoproteins were determined by sodium dodecyl sulphate gel electrophoresis and autoradiography. In the absence of ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), there was little phosphorylation due to the high level of ATPase activity. Phosphorylation of three proteins of 64,000 daltons (*E1*), 42,000 daltons (*E2*), and 20,000 daltons (*E3*) was observed in the presence of 1 mM EGTA. Phosphorylation of these proteins was cAMP-independent, hydroxylamine-resistant, and was seen without the addition of protein kinase. In the presence of HgCl₂ (2.5 mM) or sodium deoxycholate (1%) no protein phosphorylation was observed. Protein *E1* was heavily phosphorylated in the presence of 200 mM KCl, while its phosphorylation was inhibited by 20 μM sodium dantrolene, an inhibitor of Ca²⁺ release. Phosphoprotein *E3* was found in light and heavy sarcoplasmic reticulum vesicles while *E1* and *E2* were found only in heavy vesicles. The phosphoprotein *E2* had the properties of an intrinsic membrane protein while the protein *E1* behaved as an extrinsic membrane protein. Proteins *E2* and *E3* corresponded in mobility to minor sarcoplasmic reticulum proteins while *E1* had the same mobility as calsequestrin. The presence of high calcium (5 mM) during electrophore-

sis caused calsequestrin to run at a lower molecular weight (~56,000 instead of 64,000 daltons), and correspondingly the phosphoprotein *E1* ran at a lower molecular weight. Finally, calsequestrin purified by a double gel electrophoresis method has been shown to be phosphorylated.

It is generally accepted that depolarization of the transverse tubular system of skeletal muscle initiates the release of calcium from the terminal cisternae of the sarcoplasmic reticulum (Ebashi & Endo, 1968; Endo, 1977; Fuchs, 1974; Sandow, 1970). In recent years there have been several mechanisms proposed for the link between the depolarization of the walls and of the T-tubule and the release of calcium from the sarcoplasmic reticulum (Ebashi & Endo, 1968; Endo, 1977), but it still remains one of the least understood processes in muscle contraction. The skeletal muscle membranes directly involved in excitation-contraction coupling are the transverse tubular membrane and the junctional sarcoplasmic reticulum membrane (Franzini-Armstrong, 1975). The morphological and chemical differences of the longitudinal sarcoplasmic reticulum and the terminal cisternae are consistent with the hypothesis that the longitudinal SR contains, predominantly, the calcium pump protein and is responsible for calcium uptake resulting in relaxation, and that the terminal cisternae, is responsible for the calcium release to initiate contraction and contains one or more membrane proteins besides the calcium pump protein (Franzini-Armstrong, 1975; Meissner, 1975; Campbell, Armstrong & Shamoo, 1980).

It is now becoming increasingly evident that the phosphorylation of membranes by protein kinases

* Present Address: Banting and Best Department of Medical Research, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, Canada.

** Present Address and to whom reprint requests should be made: Department of Biological Chemistry, University of Maryland, 660 West Redwood Street, Baltimore, Maryland 21202.

(*c*AMP-dependent or -independent) may represent a mechanism for controlling calcium movements across membranes. Rudolph and Greengard (1975) have shown that beta-adrenergic agents and *c*AMP regulate the membrane permeability and protein phosphorylation in the avian erythrocyte. Altered phosphorylation of membrane channels has been suggested as a possible mechanism linking *c*AMP to the increased slow inward current in the heart surface membrane (Tsien, 1978). Krueger, Forn and Greengard (1977) have shown that depolarization of synaptosomes induces the phosphorylation of specific proteins. The phosphorylation requires the influx of calcium ions and is not affected by cyclic nucleotides. The action of beta-adrenergic agonists on muscle seems to mediate through both soluble and membrane-bound protein kinases.

In cardiac muscle it has been shown that beta-adrenergic agonists cause an increase in *c*AMP which causes a *c*AMP-dependent protein kinase to phosphorylate a 22,000-dalton SR protein (Kirchberger & Tada, 1976). This phosphorylation has been connected with increased calcium uptake by cardiac sarcoplasmic reticulum vesicles. It has been reported that skeletal muscle SR has little or no phosphorylation (Kirchberger & Tada, 1976). In previous work it has been shown that the preparation of heavy SR vesicles derived from the terminal cisternae of the sarcoplasmic reticulum contain the junctional sarcoplasmic reticulum membrane (Meissner, 1975; Campbell et al., 1980). Heavy SR vesicles have been shown to have intact "SR feet" on the surface of their membrane, whereas the "SR feet" are absent in the vesicles which have been washed with KCl (Campbell et al., 1980). Recently, it has been found that the SR protein, calsequestrin, has the properties of a protein kinase (Varanyi & Herlmeyer, 1979). The purpose of our investigation was to characterize the endogenous phosphorylation of heavy sarcoplasmic reticulum proteins and to identify the phosphorylated proteins. A preliminary report of this work was presented (Campbell & Shamoo, 1978).

Materials and Methods

Sarcoplasmic Reticulum Preparations

Light and heavy sarcoplasmic reticulum vesicles (LSR, HSR) were isolated according to the method of Campbell et al. (1980). Sucrose-washed vesicles were referred to as LSR-sucrose and HSR-sucrose. KCl washed vesicles are referred to as LSR-KCl and HSR-KCl. Intrinsic and extrinsic membrane proteins were examined using deoxycholate treatment of SR vesicles according to the method of MacLennan (1970). Mg^{2+} -ATPase and $Ca^{2+} + Mg^{2+}$ ATPase activity were measured according to MacLennan (1970). Protein

was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using a 10% slab gel with a 3% stacking gel. SR samples were solubilized in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 1 mM EDTA, 5% mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue (SDS-sample buffer) at a protein concentration of 1 mg/ml for 2 min at 100 °C followed by 2 hr at 37 °C. After electrophoresis the gel was stained, destained, and dried on Whatman No. 5 paper.

SDS polyacrylamide gel electrophoresis was also carried out according to the method of Weber and Osborn (1968). Apparent molecular weights were calculated from a graph of relative mobilities vs. log of mol wt for each gel system. The following proteins, obtained from Sigma Chemical Co., were used as mol wt standards, rabbit muscle phosphorylase (94,600), bovine liver catalase (57,500), bovine carbonic anhydrase (30,000), and soybean trypsin inhibitor (21,500). Red blood cell spectrin (220,000 and 240,000) and rabbit muscle actin (43,000) were a generous gift from Dr. F. Kirkpatrick.

Phosphorylation of Sarcoplasmic Reticulum Vesicles

The standard reaction mixture contained 5 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES (pH 7.4), 100–200 μ g of SR protein and 10 μ M ATP- γ - ^{32}P (20 Ci/mmol, Amersham/Searle) in a final volume of 0.1 ml. The SR vesicles were incubated at room temperature for 30 min in a reaction mixture prior to initiation of phosphorylation. Phosphorylation was initiated by the addition of ^{32}P -ATP. The reaction was terminated by the addition of 0.05 ml of SDS sample buffer (3X concentrated) and heated as described. Aliquots (0.05 ml) from each reaction mixture were subjected to SDS gel electrophoresis as described. The dried slab gel was placed on Kodak NS 2T film for 2–7 days. The resulting autoradiograph revealed those proteins into which ^{32}P had been incorporated. The optical density of the bands on the film was measured on a Beckman densitometer, and the areas under the peaks of the optical density tracings were used as a quantitative measure of incorporation of ^{32}P into SR proteins. Characterization of the phosphorylation involved the addition of reagents to the standard reaction mixture to investigate their effects of phosphorylation.

Isolation of Calsequestrin

Calsequestrin was purified according to the method of MacLennan and Wong (1971) from phosphorylated SR vesicles. Calsequestrin was partially purified by the method of Ikemoto, Bhatnagar, and Gergely (1971) using calcium precipitation.

Calsequestrin was also purified using a double electrophoresis system which makes use of the fact that calsequestrin runs as a 44,000-dalton protein on Weber and Osborn (1968) SDS-gels and as a 64,000-dalton protein on Laemmli (1970) SDS gels. Any minor contamination of calsequestrin on one gel system would be removed on the second gel system. Phosphorylated heavy vesicles were solubilized and run on a 7.5% Weber and Osborn (1968) disc gel. After staining and destaining, calsequestrin, which ran at 44,000 daltons, was cut out from the gel, washed with water to remove acetic acid, and homogenized in Laemmli sample buffer. The homogenate was then run on a Laemmli (1970) slab gel, which revealed a single band of 64,000 daltons.

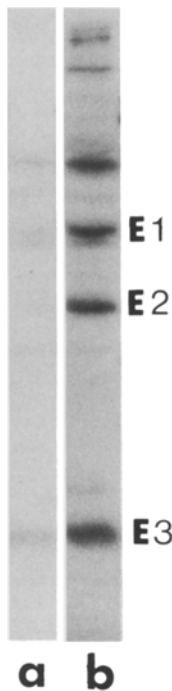


Fig. 1. Effect of EGTA on the endogenous phosphorylation of heavy sarcoplasmic reticulum. Heavy vesicles were preincubated with 5 mM $MgCl_2$ 10 mM HEPES (pH 7.4) in the presence or absence of 1 mM EGTA for 30 min at room temperature. Phosphorylation was initiated by the addition of $10 \mu M$ ^{32}P -ATP, terminated by the addition of SDS sample buffer after 10 min, and the samples subjected to SDS-gel electrophoresis according to Laemmli (1970). The slab gel was stained, dried, and exposed to X-ray film to produce the autoradiogram shown. Fifty micrograms of protein were run in each lane of the slab gel. (a): No EGTA. (b): 1 mM EGTA. Phosphorylated proteins *E1* (64,000 daltons), *E2* (42,000 daltons), and *E3* (20,000 daltons) are indicated. The higher mol wt proteins that are phosphorylated are probably proteins involved in glycolysis (see text)

Results

Characterization of Heavy Sarcoplasmic Reticulum Phosphorylation

An autoradiogram illustrating the incorporation of ^{32}P into heavy SR proteins is shown in Fig. 1. Heavy SR had been preincubated in 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4) plus any added reagent for 30 min at room temperature. The phosphorylation was initiated by γ - ^{32}P -ATP and terminated after 10 min by the addition of SDS sample buffer. When heavy SR vesicles were incubated in the standard reaction mixture there was very little ^{32}P incorporation into heavy SR proteins (Fig. 1a). The addition of 1 mM EGTA

¹ Abbreviations: SR, sarcoplasmic reticulum vesicles; ATPase, adenosine triphosphatase, SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β -aminoethyl ether) N' , N' -tetraacetic acid; HSR, heavy sarcoplasmic reticulum vesicles.

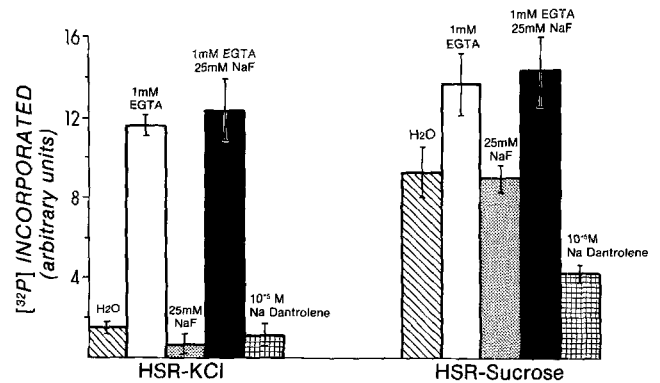


Fig. 2. ^{32}P -incorporation into *E1* for KCl-washed heavy SR and sucrose-washed heavy SR. Phosphorylation was carried out for 10 min in the presence of 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), $10 \mu M$ ^{32}P -ATP and the added reagent. Left to right: H_2O , 1 mM EGTA, 25 mM NaF, 1 mM EGTA + 25 mM NaF, 10^{-5} M Na dantrolene. Phosphorylation was initiated by the addition of ^{32}P -ATP, terminated by SDS, and samples subjected to Laemmli (1970) SDS-PAGE. The resultant autoradiogram was scanned with a Beckman densitometer, and the area under the band *E1* was measured

to the reaction mixture dramatically stimulated the phosphorylation of three specific proteins, *E1* (64,000 daltons), *E2* (42,000 daltons), and *E3* (20,000 daltons) (Fig. 1b).

The apparent requirements of EGTA for the phosphorylation of heavy vesicles is due to the high Ca^{2+} ATPase activity, $1.8 \mu mol P_i/mg/min$ in the absence of EGTA as compared to $<0.07 \mu mol P_i/mg/min$ in the presence of 1 mM EGTA. Therefore, under the conditions of phosphorylation ($10 \mu M$ ATP), the ^{32}P - γ -ATP would have lasted only 3 sec in the absence of EGTA and greater than 80 sec in the presence of 1 mM EGTA. Apparent molecular weights of the phosphorylated proteins were determined from the semi-log plot of mol wt vs. mobility. The higher mol wt phosphoproteins seen in Fig. 1b have been tentatively identified by their molecular weights as glycogen debranching enzyme (160,000 daltons), phosphorylase kinase (128,000 and 145,000 daltons), phosphorylase *a* (94,600 daltons), and glycogen synthetase (88,000 daltons). It should be noted that no phosphorylation was observed when the SR vesicles were solubilized with SDS prior to the addition of ATP- γ - ^{32}P . Also, the treatment of SDS-solubilized samples with 0.8 M hydroxylamine at 30 °C for 30 min caused no decrease in the level of phosphorylation. This result was expected since the method of determining ^{32}P incorporation using SDS gel electrophoresis, followed by staining and extensive destaining in 10% acetic acid, should hydrolyze acyl phosphate bonds and leave phosphate ester bonds intact.

Quantitative results of the phosphorylation of protein *E1* in two types of heavy vesicles are given in Fig. 2. HSR-KCl vesicles which were washed with

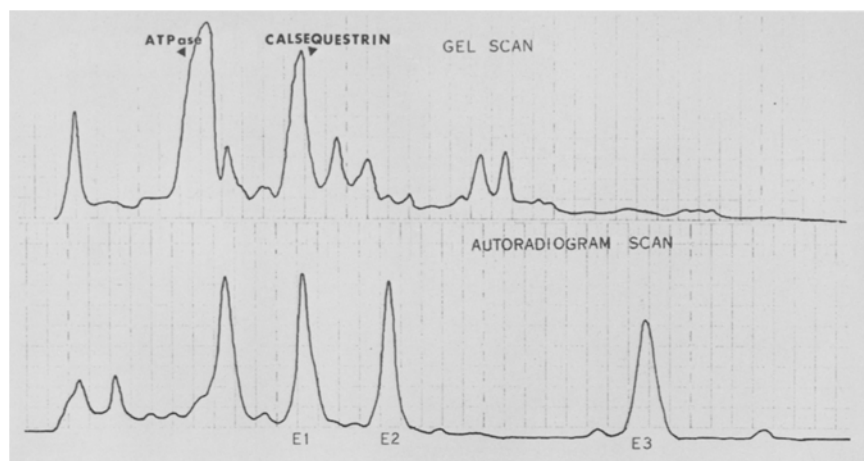


Fig. 3. Optical density scans of Coomassie blue staining of heavy SR proteins run on a Laemmli (1970) slab gel (upper scan) and of an autoradiogram of the same gel lane (lower scan) showing the ^{32}P -phosphorylated proteins, *E1*, *E2* and *E3*. Band *E1* corresponds in mobility to the SR protein calsequestrin, while *E2* and *E3* correspond to only minor HSR proteins. The phosphorylated protein running slightly faster than the ATPase is phosphorylase

0.6 M KCl. They had a low level of endogenous calcium and magnesium and high ATPase activity (Campbell et al., 1980). HSR-sucrose vesicles had a high level of endogenous calcium and magnesium and a low level of ATPase activity. A sixfold increase in ^{32}P incorporation into *E1* was observed when 1 mM EGTA was added to the standard reaction mixture in the case of HSR-KCl vesicles. This increase was also true for the phosphorylation of *E2* and *E3*. NaF (25 mM), alone, caused little change in the level of *E1* or *E3* phosphorylation. In HSR-sucrose vesicles the level of *E1* phosphorylation in the absence of EGTA was relatively high when compared to that of HSR-KCl vesicles, but 1 mM EGTA did cause a 45% increase in ^{32}P incorporation into *E1*. NaF (25 mM), alone, had no effect, while Na dantrolene (10^{-5} M), an inhibitor of Ca^{2+} release (Desmedt & Hainaut, 1977), reduced the phosphorylation of *E1* by 60% in the HSR-sucrose vesicles.

Previous studies have been unsuccessful in measuring phosphorylation of skeletal muscle sarcoplasmic reticulum vesicles (Kirchberger & Tada, 1976). In this study we were successful probably due to the use of heavy SR vesicles, which have a high level of phosphorylation, and a very low level of basal ATPase activity, and because the technique of autoradiography is much more sensitive than counting slices of gels to determine ^{32}P incorporation. Also, SR vesicles isolated without a sucrose gradient contain such large amounts of glycolytic enzymes that they are the only phosphoproteins seen on the SDS gel.

Figure 3 shows an optical density scan of a Coomassie blue stained gel (upper scan) and the corresponding autoradiogram (lower scan) after phosphorylated heavy SR vesicles were run on a Laemmli (1970) SDS gel. The major proteins in the heavy SR vesicles are the Ca^{2+} ATPase (100,000 daltons) and calsequestrin (64,000 daltons). ^{32}P phosphorylated

proteins *E1*, *E2* and *E3* are indicated in the scan of the autoradiogram. Protein *E2* and *E3* corresponded to minor SR proteins in their mobility in the gel, while phosphoprotein *E1* corresponded in mobility to calsequestrin.

Figure 4 shows the results of HSR-KCl and HSR sucrose phosphorylation in the presence of 1 mM EGTA, 5 mM MgCl_2 , 10 mM HEPES (pH 7.4) and 10 μM $\text{ATP-}\gamma\text{-}^{32}\text{P}$ from 15 sec to 10 min. Phosphorylated proteins *E1*, *E2* and *E3* were phosphorylated faster in the HSR-KCl vesicles, but after 10 min both types of heavy vesicles had the same level of phosphorylation in protein *E1* and *E3*. HSR-KCl (Fig. 4, # 5) contained less of the high mol wt phosphorylated proteins than the HSR-sucrose vesicles (Fig. 4, # 6). This is consistent with the fact that KCl washing removes glycolytic enzymes from the SR vesicles. It should be noted that the band *E1* appears as a doublet in Fig. 4 and that calsequestrin has been reported to exist in two forms which differ in mol wt by 6% (MacLennan, 1974). Light SR vesicles (not shown) in the absence of EGTA contained no phosphorylated proteins while in the presence of 1 mM EGTA contained very faint phosphorylation bands of *E1* and *E2*, and equal amount of *E3* when compared to heavy vesicles.

The addition of 100 mM KCl to the reaction mixture caused protein *E1* to become heavily phosphorylated (Fig. 5*a'*), while the level of phosphorylation in *E2* and *E3* was greatly reduced. The phosphorylated *E1* (Fig. 5*a'*) and calsequestrin (Fig. 5*a*) ran at the same position on the Laemmli slab gel and covered the same area on the gel. ^{32}P incorporation into protein *E1* in the presence of KCl was shown to peak around 2 min (which is approximately the time all the $^{32}\text{P-}\gamma\text{-ATP}$ would be hydrolyzed) and then decay. The addition of 25 mM NaF caused the level of ^{32}P -incorporation to remain constant after 2 min.

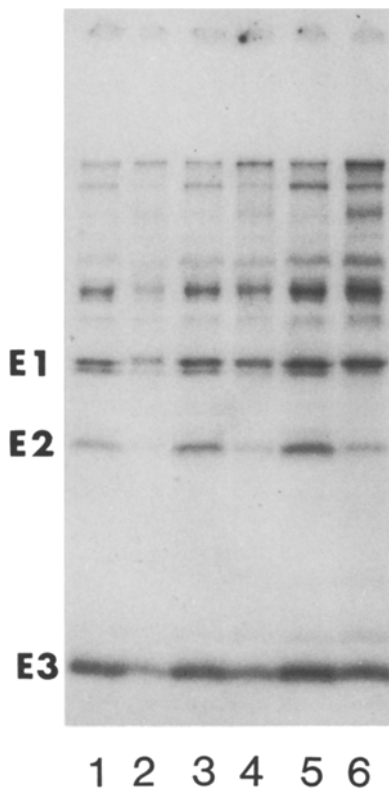


Fig. 4. Time course of phosphorylation of KCl-washed heavy SR and sucrose-washed heavy SR. Vesicles were phosphorylated in the presence of 1 mM EGTA, 5 mM MgCl₂, 10 mM HEPES (pH 7.4), and 10 μM ATP-γ-³²P, and then subjected to SDS-PAGE. The resultant autoradiogram is shown. KCl-washed vesicles (1, 3, 5) and sucrose-washed vesicles (2, 4, 6); 15 sec (1, 2), 1 min (3, 4) and 10 min (5, 6) phosphorylations. Proteins E1, E2 and E3 are indicated. Note the phosphorylated protein E1 appears as a doublet on this gel

Table 1 gives a summary of the requirements for protein E1 phosphorylation. We have shown that Mg²⁺ and EGTA are required for phosphorylation of E1. EGTA is required because the Ca²⁺ ATPase must be inhibited. Mg²⁺ was found to be required because in the presence of 25 mM EDTA no phosphorylation of E1 is observed. NaF stimulates the phosphorylation of E1 by inhibiting dephosphorylation. Triton X-100 (0.2%) stimulated the phosphorylation of E1 by over fourfold, probably by making the SR membrane leaky so ³²P-γ-ATP

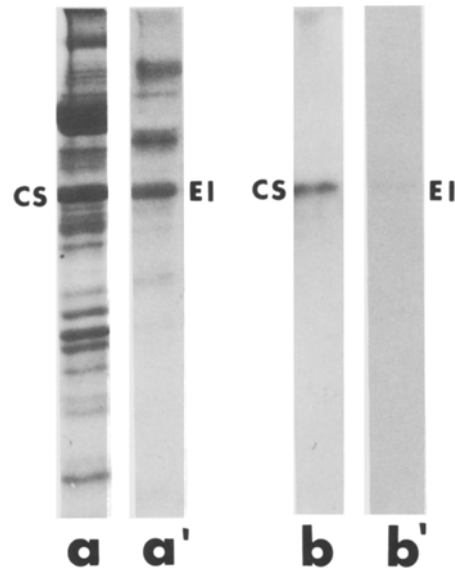


Fig. 5. Coomassie blue staining (a) and corresponding autoradiogram (a') of phosphorylated vesicles subjected to SDS-PAGE. Heavy vesicles were phosphorylated in the presence of 200 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.4) and 10 μM ATP-γ-³²P for 10 min. In the presence of high KCl protein E1 was heavily phosphorylated while proteins E2 and E3 were barely visible. Calsequestrin (CS) and band E1 have the same mobility. Coomassie blue staining (b) and corresponding autoradiogram (b') of isolated calsequestrin. Calsequestrin was purified by double gel electrophoresis according to Weber and Osborn first and Laemmli second. Vesicles were phosphorylated and then run on 7.4% Weber and Osborn disc gels, and the gels were stained and destained as described. The band running at 44,000 daltons, which is calsequestrin, was cut out from the gel, washed with water to remove acetic acid, and homogenized in Laemmli SDS sample buffer (see Materials and Methods). The homogenate was placed on a Laemmli slab gel and electrophoresis as described. The stained gel (b) reveals a band of approximately 64,000 (CS, calsequestrin) while the resultant autoradiogram reveals phosphorylated protein E1 (b')

was more accessible to protein E1. Na dantrolene, as in the absence of EGTA, caused an inhibition of the phosphorylation of E1 by over 50%. HgCl₂ (2.5 mM) and 1% deoxycholate abolished the phosphorylation of E1 and the other phosphorylated proteins. cAMP left the level of E1 phosphorylation unchanged, while it increased phosphorylation of phosphorylase kinase, phosphorylase, and glycogen synthetase. Two other SR proteins of mol wt 56,000

Table 1. Conditions for protein E1 phosphorylation

Requirements	Stimulatory	Inhibitory	No effect
Mg ²⁺	NaF (25 mM)	Dantrolene (10 ⁻⁵ M)	Alkaline phosphatase
EGTA (to inhibit ATPase activity)	Triton X-100 (0.2%)	HgCl ₂ (2.5 mM)	cAMP (10 ⁻⁶ to 10 ⁻⁵ M)
	K ⁺ (200 mM)	Deoxycholate (1%)	Hydroxylamine (0.8 M)

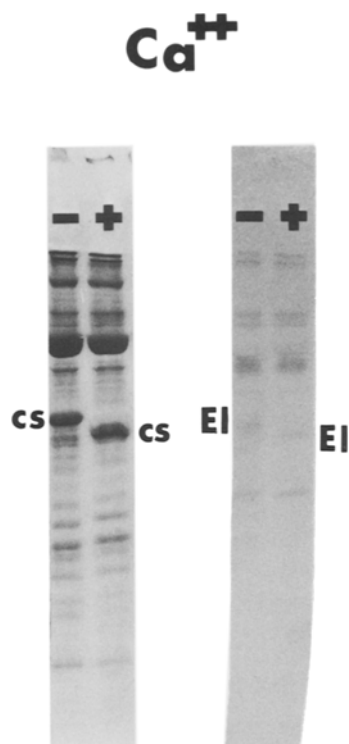


Fig. 6. Effect of Ca^{2+} on the mobility of calsequestrin (CS) and phosphoprotein *E1* in Laemmli SDS gels. Vesicles were incubated and phosphorylated in the presence of the standard reaction mixture plus 1 mM EGTA and 0.5 mM CaCl_2 (-), or 1 mM EGTA and 5 mM CaCl_2 (+), and were terminated by an equal volume of SDS sample and run on a Laemmli SDS-gel. The Coomassie blue stained gels (left) shows that calsequestrin moved at a faster mobility in the presence of mM Ca^{2+} . Its apparent mol wt changed from 64,000 to 56,000 daltons. The resultant autoradiogram (right) shows that *E1* also changed its mobility in the presence of mM Ca^{2+} . Its apparent mol wt went from 64,000 to 56,000 daltons

and 28,000 daltons were seen to be phosphorylated in the presence of *cAMP* (10^{-5} M). Alkaline phosphatase had no effect on the phosphorylation of *E1*, but it did reduce the amount of ^{32}P incorporated into *E2*, phosphorylase kinase, and phosphorylase *a*.

Purification of Phosphorylated Calsequestrin

In an attempt to conclusively identify protein *E1* as calsequestrin, we have tried to purify calsequestrin from phosphorylated vesicles and show that it is phosphorylated. Purification of calsequestrin by the method of MacLennan and Wong (1971) involves the treatment of SR with deoxycholate to remove the extrinsic proteins, extensive dialysis against 5 mM Tris HCl, pH 7.5, followed by DEAE and hydroxylapatite chromatography. The results of deoxycholate

treatment of vesicles revealed that *E1* could be removed by deoxycholate in the presence of 1 M KCl while *E2* and *E3* remained in the membrane. This indicates that *E1* is an extrinsic membrane protein and *E2* and *E3* are intrinsic membrane proteins. After dialysis and DEAE chromatography we were not able to find any remaining ^{32}P in calsequestrin. This was probably due to dephosphorylation of calsequestrin during the long isolation. Isolation of partially purified calsequestrin by the calcium precipitation method of Ikemoto et al. (1971) revealed ^{32}P incorporation in the calsequestrin band, but we were unable to purify it further without losing the phosphoryl groups.

The most successful method of isolation of phosphorylated calsequestrin involved a double gel electrophoresis method. This method used the fact that calsequestrin ran as a 44,000-dalton protein on Weber and Osborn gels and a 64,000-dalton protein on Laemmli gels. Phosphorylated vesicles were first run on a 7.5% Weber and Osborn disc gel, and the band corresponding to calsequestrin was cut out, washed with water, and homogenized in Laemmli SDS sample buffer. The standard Laemmli gel (Fig. 5*b*) revealed a single band of 64,000 daltons corresponding to calsequestrin while the resulting autoradiogram revealed (Fig. 5*b'*) phosphorylated protein *E1*. This method was most successful because it was the quickest and calsequestrin had little time to dephosphorylate.

Figure 6 shows the effect of calcium on the mobility of calsequestrin and *E1* in SDS gels. In our studies on the effect of calcium on the phosphorylation of *E1* we found that at increasing calcium concentrations the phosphorylation of *E1* was decreased. An interesting finding in these studies was that 5 mM Ca^{2+} can change the mobility of calsequestrin in an SDS gel. The Coomassie blue stained gel (left, Fig. 6) showed that calsequestrin moved at a faster mobility in the presence of 5 mM Ca^{2+} . Its apparent mol wt went from 64,000 to 56,000 daltons. The resultant autoradiogram showed that *E1* also changed its mobility to 56,000 daltons. This is further proof that the phosphorylated protein *E1* is calsequestrin.

Discussion

The heavy sarcoplasmic reticulum proteins which are phosphorylated by an endogeneous protein kinase can be broken up into three classes. The first class are the proteins which are involved in glycolysis. They have been identified as glycogen debranching enzyme, phosphorylase kinase, phosphorylase, and glycogen synthetase. The second class of phosphorylated pro-

teins are those whose phosphorylation is *cAMP* dependent or stimulated by *cAMP*. The phosphorylation of the glycolytic enzymes was stimulated by the presence of 10^{-6} to 10^{-5} M *cAMP*. The only other proteins affected by *cAMP* were a protein of mol wt 28,000 daltons, seen only in the presence of *cAMP* (10^{-5} M), and a protein of 56,000 daltons, whose phosphorylation is stimulated in the presence of *cAMP* (10^{-5} M).

The third class of phosphorylated proteins are *E1*, *E2*, and *E3*. The phosphorylation of protein *E3*, 20,000 daltons, had been found in all SR fractions. Its phosphorylation was extremely fast and within 1 min it reaches its maximum level of phosphorylation. It has the properties of an intrinsic membrane protein when subjected to deoxycholate treatment and was different from phospholamban (Kirchberger & Tada, 1976) in that it was not stimulated by *cAMP*, did not require added protein kinase. The phosphorylation of band *E2*, 42,000 daltons, was only found in the heavy vesicles. It had the unique property of being phosphorylated in the presence of 25 mM EDTA. It also had the properties of an intrinsic membrane protein when subjected to deoxycholate treatment. Treatment of phosphorylated vesicles with alkaline phosphatase completely removed the phosphorylation of *E2*, indicating it had an external phosphorylation site.

The phosphorylated protein *E1* (64,000 daltons) was predominantly found in heavy vesicles, and it was the only phosphorylated protein which corresponded to a major protein constituent of the heavy vesicles. The treatment of heavy vesicles with deoxycholate revealed that *E1* was an extrinsic membrane protein. The data supporting the identification of *E1* as calsequestrin are (i) *E1* and calsequestrin have the same mobility on SDS gels and this mobility is changed in the presence of mM Ca^{2+} ; (ii) *E1* and calsequestrin are extrinsic membrane proteins; (iii) both are precipitated by high Ca^{2+} after the vesicles are solubilized by Triton X-100; and (iv) isolation of calsequestrin using a double gel electrophoresis system has shown that *E1* and calsequestrin are the same protein.

The recent finding that calsequestrin has properties of a protein kinase (Varsanyi & Herlmeyer, 1979) is a possible reason why we find calsequestrin phosphorylated. In addition to being a protein kinase, calsequestrin might also be a substrate for protein kinase. In our experiments we are not able to distinguish between the two possibilities.

The findings presented here have shown the existence of an endogenous membrane protein kinase and substrate proteins in heavy SR vesicles. Whether

the phosphorylation and dephosphorylation of these proteins is involved in the mechanism of excitation-contraction coupling is an interesting possibility. The location of the phosphorylation in vesicles which are derived from the terminal cisternae and the inhibition of phosphorylation of *E1* by dantrolene (an inhibitor of Ca^{2+} release) lends support to this possibility. Combined studies on calcium release from heavy SR vesicles and phosphorylation of heavy SR vesicles may lead to some interesting findings on the control of Ca^{2+} permeability of the terminal cisternae of the sarcoplasmic reticulum.

This paper is based on work performed under contract with the U.S. Department of Energy at the University of Rochester, Department of Radiation, Biology and Biophysics, and has been assigned Report No. UR-3420-1744. This paper was also supported by Grant # 1 R01 18892 from the N.I.H. Program Project ES-10248 from NIEHS, and by a grant from MDA. Kevin Campbell was Elon Huntington Hooker Graduate Fellow. This work was submitted (by K.P.C.) to the Graduate School of the University of Rochester in partial fulfillment of the requirements for a Ph.D. degree. Adil E. Shamoo is an Established Investigator of American Heart Association, Inc.

References

- Campbell, K.P., Franzini-Armstrong, C., Shamoo, A.E. 1980. Further characterization of light and sarcoplasmic reticulum vesicles. Identification of the "SR Feet" with heavy sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* (in press)
- Campbell, K.P., Shamoo, A.E. 1978. Intrinsic phosphorylation of heavy sarcoplasmic reticulum vesicles. *Biophys. J.* **21**:47a
- Desmedt, J.E., Hainaut, J. 1977. Inhibition of intracellular release of calcium by dantrolene in barnacle giant muscle fibres. *J. Physiol. (London)* **265**:565
- Ebashi, S., Endo, M. 1968. Calcium ions and muscle contraction. *Progr. Biophys. Mol. Biol.* **18**:123
- Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**:171
- Franzini-Armstrong, C. 1975. Membrane particles and transmission at the triad. *Fed. Proc.* **34**:1382
- Fuchs, F. 1974. Striated Muscle. *Annu. Rev. Physiol.* **36**:461
- Ikemoto, N., Bhatnagar, G.M., Gergely, J. 1971. Fractionation of solubilized sarcoplasmic reticulum. *Biochim. Biophys. Acta* **44**:1510
- Kirchberger, M.S., Tada, M. 1976. Effects of *cAMP* protein kinase on SR isolated from cardiac and slow and fast contracting skeletal muscles. *J. Biol. Chem.* **251**:725
- Krueger, B.K., Forn, J., Greengard, P. 1977. Depolarization induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.* **252**:2764
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265
- MacLennan, D.H. 1970. Purification and properties of an ATPase from sarcoplasmic reticulum. *J. Biol. Chem.* **245**:4508

- MacLennan, D.H. 1974. Isolation of a second form of Calsequestrin. *J. Biol. Chem.* **249**:980
- MacLennan, D.H., Wong, P.T.S. 1971. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc. Nat. Acad. Sci. USA* **68**:1231
- Meissner, G. 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **389**:51
- Rudolph, S.A., Greengard, P. 1975. Regulation of protein phosphorylation and membrane permeability by beta adrenergic agents and cAMP in the avian erythrocyte. *J. Biol. Chem.* **249**:5684
- Sandow, A. 1970. Skeletal Muscle. *Annu. Rev. Physiol.* **17**:87
- Tsien, R.W. 1978. Cyclic AMP and contractile activity in heart. *In: Advances in Cyclic Nucleotide Research*. P. Greengard and G. Robinson, editors. Vol. 8, p. 363. Raven Press, New York
- Varsanyi, M., Herlmeyer, M.G. 1979. The protein kinase properties of calsequestrin. *FEBS Lett.* **103**:35
- Weber, K., Osborn, M. 1968. The reliability of molecular weight determinations by SDS-gel electrophoresis. *J. Biol. Chem.* **244**:4406

Received 12 March 1980; revised 8 May 1980