Specific Association of Calmodulin-Dependent Protein Kinase and Related Substrates with the Junctional Sarcoplasmic Reticulum of Skeletal Muscle†

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ABSTRACT: A systematic study of protein kinase activity and phosphorylation of membrane proteins by ATP was carried out with vesicular fragments of longitudinal tubules (light SR) and junctional terminal cisternae (JTC) derived from skeletal muscle sarcoplasmic reticulum (SR). Following incubation of JTC with ATP, a 170 000-Da glycoprotein, a 97 500-Da protein (glycogen phosphorylase), and a 55 000–60 000-Da doublet (containing calmodulin-dependent protein kinase subunit) underwent phosphorylation. Addition of calmodulin in the presence of Ca^{2+} (with no added protein kinase) produced a 10-fold increase of phosphorylation involving numerous JTC proteins, including the large ($\sim$450 000 Da) ryanodine receptor protein. Calmodulin-dependent phosphorylation of the ryanodine receptor protein was unambiguously demonstrated by Western blot analysis. The specificity of these findings was demonstrated by much lower levels of calmodulin-dependent phosphorylation in light SR as compared to JTC, and by much lower cyclic AMP dependent kinase activity in both JTC and light SR. These observations indicate that the purified JTC contain membrane-bound calmodulin-dependent protein kinase that undergoes autophosphorylation and catalyzes phosphorylation of various membrane proteins. Protein dephosphorylation was very slow in the absence of added phosphatases, but was accelerated by the addition of phosphatase 1 and 2A (catalytic subunit) in the absence of Ca^{2+}, and calcineurin in the presence of Ca^{2+}. Therefore, in the muscle fiber, dephosphorylation of SR proteins relies on cytoplasmic phosphatases. No significant effect of protein phosphorylation was detected on the Ca^{2+}-induced Ca^{2+} release exhibited by isolated JTC vesicles. However, the selective and prominent association of calmodulin-dependent protein kinase and related substrates with junctional membranes, its Ca^{2+} sensitivity, and its close proximity to the ryanodine and dihydropyridine receptor Ca^{2+} channels suggest that this phosphorylation system is involved in regulation of functions linked to these structures.

Kinase-dependent protein phosphorylation appears to have a significant regulatory role in many cell functions (Krebs, 1986), including myosin activation of smooth muscle contraction (Shenolikar, 1988), voltage-dependent calcium channel function (Reuter, 1974; Tsien & Weingart, 1976; Curtis & Catterall, 1985; Hosey et al., 1986; Imagawa et al., 1987a; Catterall et al., 1988), and neuronal signal transduction (Hemmings et al., 1989). With respect to SR, the possibility that protein phosphorylation may modulate calcium release and excitation–contraction coupling (Campbell & MacLennan, 1982; Kim & Ikemato, 1986) is of interest.

Various protein components of isolated membrane fractions (Campbell & Shamoo, 1980; Campbell & MacLennan, 1982; Chiesi & Carafoli, 1983; Seiler et al., 1984; Morii et al., 1987), and membrane-bound enzymes involved in glycogen metabolism (Campbell & Shamoo, 1980; Varsanyi & Heilmeyer, 1981; Chiesi & Carafoli, 1983), are phosphorylated by type II Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Walaas et al., 1988), phosphorylase kinase (Varsanyi & Heilmeyer, 1981), cAMP-dependent protein kinase (cAK) (Galani-Kranias et al., 1980), and an intrinsic kinase which is not stimulated by either calmodulin or cAMP (Imagawa et al., 1987b). However, the specificity of these findings is not clear, since it is not known whether one of the various kinases has a prominent role and/or is associated prevalently with any of the SR subfractions (i.e., junctional vs longitudinal SR). For this reason, we have conducted a systematic study of protein phosphorylation and dephosphorylation, using purified preparations of longitudinal and junctional SR, and comparing various kinases. With our experiments, we identify the membrane fraction endowed with high kinase activity, we establish the type of kinase and the specific activator, and we clarify the conditions for phosphorylation and dephosphorylation.

MATERIALS AND METHODS

Materials. cAMP, calcineurin (1000–3000 units/mg of protein, containing 1% protein), bovine cardiac cAMP-dependent protein kinase (phosphorylating activity 1–2 picomolar units/μg), tetralithium salt of 5′-adenylyl imidophosphate (AMP-PNP), phenyl-Sepharose CL-4B, heparin (low molecular weight) from porcine intestine, Stains-all, and protein molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO). Calmodulin and tetralithium salt of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) were from CalBiochem Co. (La Jolla, CA) and Boehringer Mannheim (Indianapolis, IN), respectively. All radioisotopes were from New England Nuclear (Beverly, MA). Ca^{2+}-independent protein phosphatase 1 and the catalytic subunit of phosphatase-2A were a generous gift from Dr. Edmond H. Fischer, Department of Biochemistry, University of Washington, Seattle.

Sarcoplasmic Reticulum Preparation. Fast twitch skeletal muscles from the hindlegs and backs of New Zealand White rabbits were used for the light and junctional sarcoplasmic reticulum preparation [R2 fraction and R4 fraction (Chu et

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al., 1988a), respectively]. In some preparations, proteolytic inhibitors were added to all media, as follows: 77 nM aprotinin, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.7 μM pepstatin A, and 0.23 mM phenylmethanesulfonyl fluoride (PMSF). Triad preparations were obtained by a modification of the method of Mitchell et al. (1983), as described by Sharp et al. (1987).

$^{45}Ca^{2+}$ Uptake. Active Ca^{2+} transport activity was assayed in a reaction medium containing 50 μg of SR protein/mL, buffered salt [80 mM KCl/20 mM Tris–MOPS (3-(*N*-morpholino)propanesulfonic acid) (pH 7)], 10 mM $MgCl_2$, and 100 μM $^{45}CaCl_2$ (specific activity ~9000 cpm/nmol). Either 1 mM Na_2ATP or 2 mM acetyl phosphate (pH 7) was added to the mixture at 23–25 °C to start the reaction. Aliquots (200 μL) containing 100 μg of SR protein were filtered through Millipore filters (0.45 μm, Bedford, MA) at serial times. The filters were washed with 2 mL of 10 mM $LaCl_3$/80 mM KCl/20 mM Tris–MOPS (pH 7) and then processed for liquid scintillation counting.

$^{45}Ca^{2+}$ Release. The time course of release was determined by filtration at serial times (Chu et al., 1988b), with the aid of a rapid filtration apparatus (Biologic, Olympia, WA) for resolution in the millisecond time scale. The SR vesicles were loaded either actively or passively with varying amounts of $^{45}Ca^{2+}$ (specific activity ~9000–15 000 cpm/nmol) at 25 °C. For active loading, 50-μg aliquots of SR protein were allowed to react for 2–5 min in 1 mL of medium containing 100 μM $^{45}CaCl_2$, 10 mM $MgCl_2$, 80 mM KCl, and 20 mM MOPS, pH 7.0, and placed on Millipore filters (0.65 μm), and the medium was filtered off. For passive loading, 1 mg of SR protein/mL was equilibrated for 2 h in a medium containing 1 mM $^{45}CaCl_2$ and buffered salt, 50-μg aliquots were diluted with 1 mL of nonrelease medium (containing 10 mM $MgCl_2$ and no Ca^{2+}) and placed on Millipore filters, and the medium was filtered off. In all cases, the loaded filters were washed with 2 mL of nonrelease medium, and release of intravesicular $^{45}Ca^{2+}$ was then started by perfusing the vesicles on the filter with a medium containing 5 μM free Ca^{2+} and buffered salt, in the presence and absence of 1 mM Na_2ATP or AMP-PNP. The nonrelease medium containing 10 mM $MgCl_2$ and no Ca^{2+} was used as a control. Following perfusion with release or nonrelease media for various time intervals, the filters were processed for liquid scintillation counting without further washing.

Phosphorylation and Dephosphorylation of Sarcoplasmic Reticulum. The reaction medium for phosphorylation of SR contained 5 mM $MgCl_2$, 80 mM KCl, 20 mM Tris–MOPS (pH 7), 0.2–0.5 mM [γ-^{32}P]ATP [specific activity (~3–7) × 10^5 cpm/nmol], and other components as specified in the figure legends. The reaction was carried out in 100 μL, containing 80–150 μg of SR protein, and was stopped by adding concentrated denaturing buffer (for gel electrophoresis), so that the final mixture contained 0.025% bromphenol blue, 1% SDS, 1.25% 2-mercaptoethanol, 0.312 mM Tris-HCl (pH 6.8), and 3.75% sucrose (Laemmli, 1970). The SR (50 μg of protein) was then loaded onto polyacrylamide gels as described below.

Dephosphorylation experiments were carried out after the SR (200 μg of protein in 100 μL) was phosphorylated as described above. Either Ca^{2+}-independent protein phosphatase or calcineurin was then added in a concentrated dephosphorylation medium (20 μL) so that the final reaction contained ~1.7 mg of SR/mL, 2.2 mM nonradioactive Na_2ATP, 5 mM $MgCl_2$, 1 mM $MnCl_2$, buffered salt, and 1 mM Tris–EGTA. When calcineurin was used, $CaCl_2$ was added to maintain the desired free [Ca^{2+}]. After a 20-min incubation at 25 °C, the reaction was stopped by adding 40 μL of the concentrated denaturing buffer, and then 50 μg of SR was applied to a gel for electrophoresis.

Determination of Base-Resistant Phosphoprotein. SR was phosphorylated as described above with [γ-^{32}P]ATP, and the reaction was stopped at the appropriate time interval by 1 mL of ice-cold 10% trichloroacetic acid/0.1 mM NaH_2PO_4/1 mM Na_2ATP. The samples were centrifuged 5 min at 5000 rpm at 4 °C in a table-top centrifuge. The supernatants were removed, 200 μL of cold 0.5 N NaOH was added to each pellet, and after 5 min, 1 mL of cold wash solution (4% perchloric acid/20 mM NaH_2PO_4/1 mM Na_2ATP) was added. The pellets were subsequently centrifuged and washed 3 times. The final pellets were dissolved in 2% SDS/0.1 N NaOH for 30 min at room temperature. Aliquots were taken for liquid scintillation counting (300 μL) and protein determination (150 μL) as described below (with protein standards also containing SDS/NaOH).

SDS–Polyacrylamide Gel Electrophoresis. Slab gel electrophoresis was carried out in an alkaline pH system (Laemmli, 1970), containing a 5–15% linear polyacrylamide gradient. The gels (1.5 mm) were routinely stained with 0.125% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid and destained in 5% methanol and 7% acetic acid. When required, Stains-all was used as described by Campbell et al. (1983) to detect glycoproteins. The molecular weight markers used were myosin (205 000), β-galactosidase (116 300), phosphorylase *b* (97 500), bovine albumin (68 000), ovalbumin (43 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 500), and α-lactalbumin (14 200), or as indicated in the figure legends. For protein identification, immunoblot strips were prepared by using light SR, triads, and JTC phosphorylated with Ca^{2+} and Ca^{2+}/calmodulin, respectively. Seventy-five micrograms of each protein sample was separated on 5–16% gradient SDS–PAGE gels and transferred to nitrocellulose. Strips were stained and developed with various antibodies and then exposed to films for comparison of immunoblot and autoradiographic bands.

Autoradiography. The stained gels were dried, exposed to Kodak XAR-5 X-ray film with a Cronex intensifying screen (New England Nuclear, Quanta III) for 20-24 h at −80 °C, unless otherwise indicated, and then processed for film developing.

Densitometry. The polyacrylamide gels and autoradiograms were scanned at 580 nm with a gel scanner equipped with a halogen light source (Isco, Model 1345, Lincoln, NE). The molecular weight was estimated from densitometry of gels, with protein molecular weight markers as standards. Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. Free Ca^{2+} concentrations were estimated according to Fabiato and Fabiato (1979).

RESULTS

Subfractions of skeletal muscle microsomes, containing vesicular fragments of longitudinal sarcoplasmic reticulum (light SR) and of junctional terminal cisternae (JTC), respectively, were subjected to phosphorylation with radiolabeled ATP, solubilized with SDS, and analyzed by electrophoresis. We first describe briefly the protein profiles of light SR and JTC, as revealed by protein stain (Figure 1A), for correlation with the autoradiograms (Figure 1B) generated by radioactive phosphate. The Ca^{2+}-ATPase, which is the enzyme responsible for active transport of Ca^{2+}, is a prominent component of both preparations (Figure 1A). On the other hand, a prominent

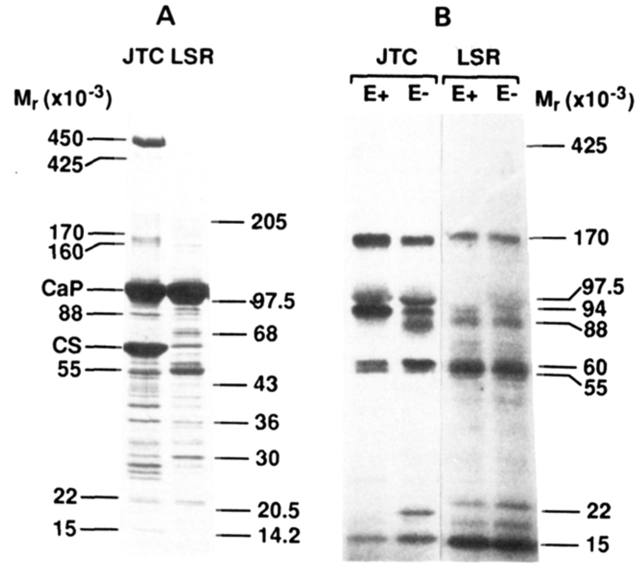


FIGURE 1: Electrophoretic patterns of JTC and light SR proteins. (A) Coomassie blue staining; (B) autoradiography following phosphorylation with 500 μM (γ^{32}P]ATP in the presence (E+) and absence of 1 mM Tris–EGTA (E–, no added Ca^{2+}). Fifty micrograms of protein was placed in each well, and electrophoresis was carried out as described under Materials and Methods. The molecular weights of protein standards used are indicated in the middle. CaP = Ca^{2+}-ATPase, CS = calsequestrin.

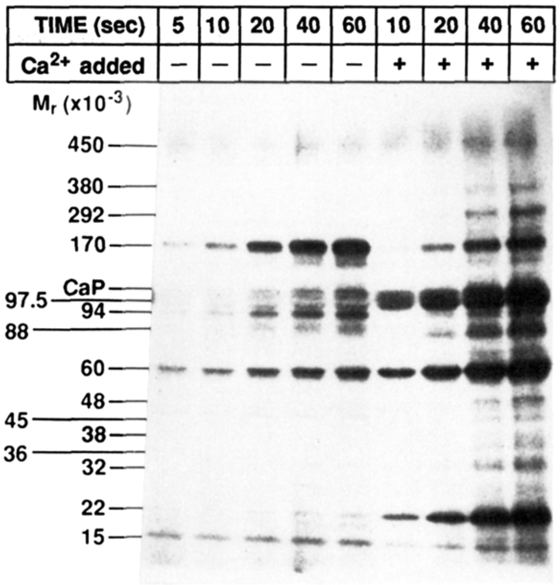


FIGURE 2: Time course of JTC protein phosphorylation. The phosphorylation reaction was carried out with 500 μM [γ-^{32}P]ATP for the time intervals noted in the figure, in the presence of 500 μM Tris–EGTA with (+) and without (–) 1.044 mM $CaCl_2$ (free [Ca^{2+}] = 521 μM), similar to the experiment shown in Figure 1.

calsequestrin band and a large protein (450 000 Da) which has been identified as the ryanodine-sensitive Ca^{2+} conductance channel (Imagawa et al., 1987b; Inui et al., 1987; Lai et al., 1988) are distinctive features of JTC.

Other minor components of JTC are shown in Figure 1A. Among them a 425 000-Da band that may be a degradation product of the ryanodine receptor protein, the 53 000-Da and 160 000-Da bands that are intrinsic glycoproteins, and a 55 000-Da band which may be the high-affinity Ca^{2+} binding protein described by Michalak et al. (1980). The 170 000-Da protein binds doxorubicin and has been shown to react with Stains-all, which is indicative of its glycoprotein character (Zorzato & Volpe, 1988). On the other hand, as the preparation inevitably contains some transverse tubule membrane contaminants, the 170 000-Da and 52 000-Da components may be subunits of the dihydropyridine receptor (Imagawa et al., 1987a). It is noteworthy that the 425 000-Da and the 170 000-Da components are less prominent in preparations containing proteolytic inhibitors. Therefore, they may contain contributions resulting from proteolysis of the ryanodine receptor protein.

The protein components phosphorylated by ATP were evidenced by autoradiography (Figure 1B). In the presence of EGTA (free [Ca^{2+}] below 10^{-8} M) and no added protein kinase, several JTC bands appeared phosphorylated, corresponding to 170 000-, 94 000-, 60 000-, and 55 000-Da proteins. Some phosphorylation was also noted on the 425 000- and 15 000-Da proteins (Figure 1B). In the absence of EGTA (~2–5 μM free Ca^{2+}), phosphorylation of the 170 000-, 94 000-, and 55 000-Da proteins was reduced, while the low level phosphorylation of 97 500- (likely glycogen phosphorylase), 88 000-, 60 000-, and 22 000-Da proteins did not change significantly. Much lower levels of phosphorylation, with the exception of a 15 000-Da protein, were found in light SR (Figure 1B).

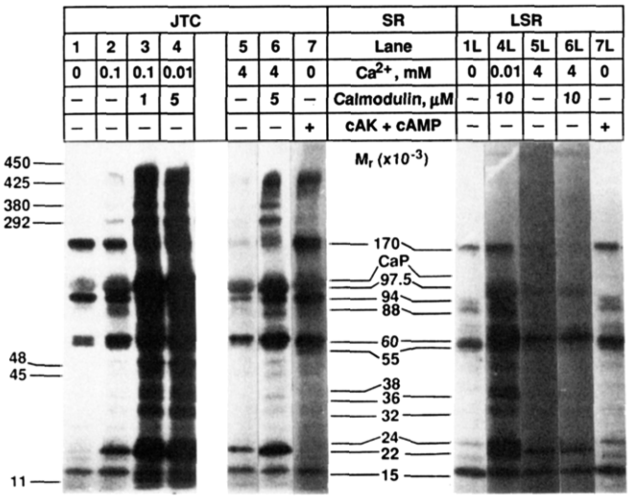
In a parallel series of experiments (not shown in figures), we studied the ATP concentration dependence of protein phosphorylation. Under prevailing conditions, 0.2–0.5 mM ATP was found to yield maximal levels of protein phosphorylation. This concentration range is optimal for most Ca^{2+}-dependent protein kinases (K_m = 0.2–0.5 mM) (Stull et al., 1986). We therefore carried out all our subsequent phosphorylation experiments with high levels of ATP (0.2–0.5 mM).

The time course of phosphorylation, in the presence and in the absence of Ca^{2+}, is shown in Figure 2. Phosphorylation reached asymptotic levels within 40–60 s at 2.5 °C. It is also shown in Figure 2 that 0.5 mM Ca^{2+} enhances phosphorylation of the 60 000-, 97 000-, and 22 000-Da proteins. On the other hand, Ca^{2+} inhibits phosphorylation of the 94 000- and 170 000-Da proteins.

These observations are indicative of considerable association of intrinsic protein kinase with the JTC preparation. It is noteworthy that strong intrinsic protein kinase activity is found in preparations of triads (Imagawa et al., 1987a), and it is possible that the activity found in our JTC preparation is due to T-tubule contaminants.

As compared with the experiments described above, we observed much higher levels of protein phosphorylation if calmodulin was added to the reaction mixture, even when no exogenous protein kinase was added (Figure 3). This effect of calmodulin was very high in the presence of Ca^{2+} in the 10^{-6}–10^{-4} M range, but was much lower if the Ca^{2+} concentration was raised to the 10^{-3} M range. Under optimal conditions, numerous proteins of JTC were phosphorylated (Figure 3), including the 450 000-Da ryanodine receptor and the 425 000-, 88 000-, 60 000-, 55 000-, 53 000-, and 30 000-Da electrophoretic bands. The Ca^{2+}-ATPase and neighboring bands that are likely enzymes involved in glycogen metabolism, such as the 145 000-Da phosphorylase kinase *a* subunit and the 135 000-Da *b* subunit (Campbell & Shamoo, 1980; Varsanyi & Heilmeyer, 1981), were also phosphorylated. A small phosphoprotein band of <11 000 Da, which may be a proteolipid (Varsanyi & Heilmeyer, 1981; Campbell & MacLennan, 1981), appeared only in the presence of added calmodulin and Ca^{2+}.

The specific-calmodulin dependent phosphorylation of the 450 000-Da ryanodine receptor protein was demonstrated unambiguously by Western blot analysis (Figure 4). Phos-



JTC							SR	LSR				
1	2	3	4	5	6	7	Lane	1L	4L	5L	6L	7L
0	0.1	0.1	0.01	4	4	0	Ca^{2+}, mM	0	0.01	4	4	0
–	–	1	5	–	5	–	Calmodulin, μM	–	10	–	10	–
–	–	–	–	–	–	+	cAK + cAMP	–	–	–	–	+

FIGURE 3: Effect of calmodulin and cAMP on phosphorylation of JTC and light SR proteins. The phosphorylation reaction was carried out as in the experiment shown in Figure 1. In this case, however, experimental variants included the Ca^{2+} concentration: 1 mM EGTA and no calcium added; 0.01, 0.1, and 4 mM Ca^{2+}, as indicated in the figure. Other experimental variants included calmodulin (17 μg or 85 μg/mL) or cAMP and cAMP-dependent protein kinase (5 μM and 100 μg/mL, respectively).

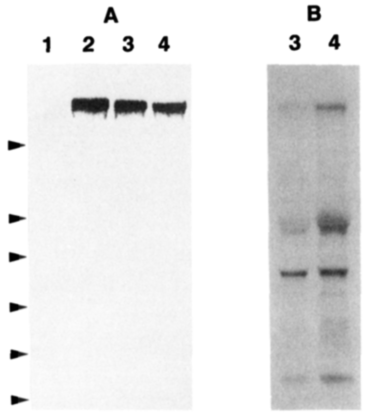


FIGURE 4: Western blot analysis of SR membrane proteins. Rabbit skeletal muscle light SR (lane 1), triads (lane 2), or isolated JTC (lanes 3 and 4) were prepared as described under Materials and Methods, subjected to SDS-PAGE on a 5–16% gradient gel, and transferred electrophoretically to nitrocellulose. Prior to gel separation, JTC samples were phosphorylated with [γ-^{32}P]ATP in the presence of 100 μM Ca^{2+} (lane 3) or 100 μM Ca^{2+} + 3 μM calmodulin (lane 4). Panel A shows immunoperoxidase staining of nitrocellulose transfers with monoclonal anti-ryanodine receptor antibody XA7. Panel B shows an autoradiogram of the same nitrocellulose transfer. Seventy-five micrograms of protein was run per lane. Molecular weight standards (224 000, 109 000, 71 800, 45 800, 28 500, and 18 400) are indicated on the left.

phorylation of the dihydropyridine receptor β subunit was also demonstrated by similar analysis (not shown).

Phosphorylation enhancement by calmodulin was also noted in light SR. However, the levels of phosphorylation were much lower (Figure 3), and possibly due to JTC contaminants in the light SR subfraction. Our experiments indicate that association of endogenous calmodulin-dependent protein kinase and related substrates is specific for the JTC. In the latter preparation, calmodulin increases the level of base resistant phosphoprotein (i.e., not including the phosphorylated intermediate of the ATPase) from 90–100 to 350–400 pmol/mg of protein (Figure 5).

As opposed to the prominent presence of calmodulin-activated protein kinase in JTC, we found very little effect of cAMP-dependent protein kinase, even after addition of both

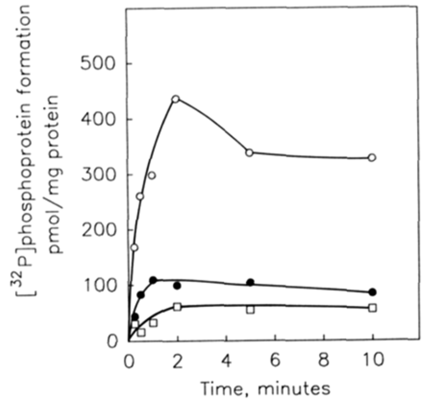


FIGURE 5: Time course of total base-resistant phosphoprotein formation in JTC. (Base-resistant phosphoprotein does not include the ATPase phosphorylated intermediate.) The phosphorylation reaction was carried out with 500 μM [γ-^{32}P]ATP as described under Materials and Methods, in the presence of 10 μM free Ca^{2+} (100 μM Tris–EGTA + 106.4 μM $CaCl_2$), with (○) and without (●) 170 μg (~10 μM) of calmodulin/mL, or in the presence of 1 mM EGTA, with 5 μM cAMP + 100 μg of cAMP-dependent kinase/mL (□).

cAMP and exogenous protein kinase (Figures 3 and 5). The 15 000-, 55 000-, 60 000-, and 425 000-Da proteins appeared to be substrates for cAK, in addition to CaMK. It is of interest that the 425 000-Da protein was phosphorylated by both added calmodulin and cAK, as also observed by Seiler et al. (1984). The 425 000-, 390 000-, 280 000-, and 150 000–170 000-Da proteins may result from proteolysis of the 450 000-Da ryanodine receptor (Imagawa et al., 1987b; Chu et al., 1988b; Lai et al., 1988).

In another group of experiments, we studied the time course of protein dephosphorylation. To this effect, following phosphorylation with radiolabeled ATP (in the presence or in the absence of Ca^{2+}), a large excess of nonradiolabeled ATP was added in the expectation that radioactive labeling of the various proteins would be reduced as the phosphorylated residues turned over and were rephosphorylated by nonradioactive ATP. We observed very little reduction of radioactivity in these chase experiments, regardless of the presence or absence of Ca^{2+} (Figure 6). Only in the case of the 22 000- and 60 000-Da proteins phosphorylated in the presence of Ca^{2+} did the chase produce a significant reduction of radioactivity (Figure 6). Such a low turnover was observed also when phosphorylation was obtained in the presence of calmodulin. In agreement with previous observations by LePeuch et al. (1979), these experiments suggest that there is very little phosphatase activity associated with the SR membrane. On the other hand, when exogenous phosphatase 1 or 2A (catalytic subunit) or calcineurin (Ballou & Fischer, 1986) was added with the nonradioactive ATP, a reduction of the radioactivity levels in the majority of the phosphorylated proteins was observed (Figure 7).

Following the experiments on SR protein phosphorylation, we examined the effects of phosphorylation and dephosphorylation on Ca^{2+} fluxes through the ryanodine-sensitive Ca^{2+} channel, in view of the fact that other Ca^{2+} channels (Reuter, 1974; Tsien & Weingart, 1976; Curtis & Catterall, 1985; Imagawa et al., 1987a; Catterall et al., 1988) can be regulated by phosphorylation. To this effect, we followed the time course of Ca^{2+}-induced Ca^{2+} release from JTC actively loaded by preincubation with ATP or acetyl phosphate, which are both effective substrates for the Ca^{2+} transport ATPase. Since ATP is a good substrate for protein kinase, while acetyl phosphate is not, we expected to obtain the release kinetics under the regulatory influence of protein phosphorylation (if any) only

SR	JTC						LSR					
Lane	1	2	3	4	5	6	7	8	9	10	11	12
Ca^{2+} present during phosphorylation	–	+	–	–	+	+	–	+	–	–	+	+
Dephosphorylation	–	–	+	+	+	+	–	–	+	+	+	+
Ca^{2+} present during Dephosphorylation			–	+	–	+			–	+	–	+

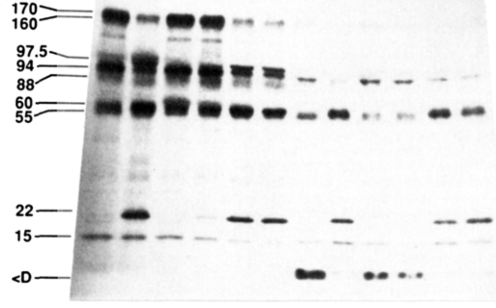


FIGURE 6: Reduction ("dephosphorylation") of radioactive [^{32}P]-phosphoprotein bands following addition ("chase") of excess nonradioactive ATP. The initial radioactive phosphorylation was carried out for 1 min as described under Materials and Methods with 500 μM [γ-^{32}P]ATP in the absence or in the presence of 100 μM free Ca^{2+} (1 mM Tris–EGTA + 1.1 mM $CaCl_2$). Excess nonradioactive ATP (7.8 mM) was then added, and the reaction was stopped after 20 min at 25 °C. This "chase" was carried out in the presence either of 5 mM EGTA (–) or of 100 μM free Ca^{2+} (+). The quenched samples were solubilized as described under Materials and Methods. The samples in lanes 1, 2, 7, and 8 did not undergo the "chase" (–). <D indicates small proteolipids below the dye front. This experiment shows that the phosphoprotein turnover is very slow and very little endogenous phosphatase is associated with the SR preparation.

from the vesicles preincubated with ATP. In fact, we saw no significant difference in the kinetics of release (Figure 8). In other experiments (not shown), we loaded the vesicles with calcium by passive equilibration after incubation for 5 min with ATPγS (to obtain phosphorylation that is not subject to rapid hydrolysis) and then started the Ca^{2+}-induced Ca^{2+} release. Even in this case, we did not observe a significant effect of phosphorylation.

Since calmodulin has a large effect on phosphorylation of JTC proteins (Figure 3), we tested its effect on Ca^{2+} fluxes. In a first set of experiments, we studied active uptake of Ca^{2+} in the presence of ATP, under conditions permitting efficient accumulation (i.e., high [Mg^{2+}]) or under conditions producing low efficiency due to passive leak through the channel (i.e., low [Mg^{2+}]). In neither case did we observe any effect of calmodulin (Figure 9). In another set of experiments, we studied directly the (passive) release of (actively) accumulated Ca^{2+}, through the Ca^{2+} (or Ca^{2+} and nucleotide) induced release mechanism. Even in this case, we found no effect of preincubation with calmodulin and ATP (Figure 10). It should be pointed out that a direct effect of calmodulin on Ca^{2+} release (in the absence of ATP) under passive Ca^{2+} loading conditions was previously observed by Meissner (1986). In this case, however, the effect was not produced through phosphorylation, since the vesicles were not exposed to ATP [see also Smith et al. (1989) and Plank et al. (1988)].

Lastly, we tested the effect of exogenous phosphatase on Ca^{2+} release. For this purpose, we used phosphatase 2A and calcineurin, which were shown to be active in dephosphorylating JTC proteins (Figure 7). However, the kinetics of Ca^{2+} release were not significantly altered by preincubation of JTC with either of these enzymes (data not shown).

DISCUSSION

Our comparative measurements of protein phosphorylation in purified subfractions of SR vesicles revealed protein kinase

1	2	3	4	5	6	7	8	Lane	9	10
E+				E–				Phosphorylation	Ca^{2+}	
–	+	+	+	–	+	●	+	Dephosphorylation	●	●
–	–	1	2A	–	–	1	2A	Phosphatase	–	CN

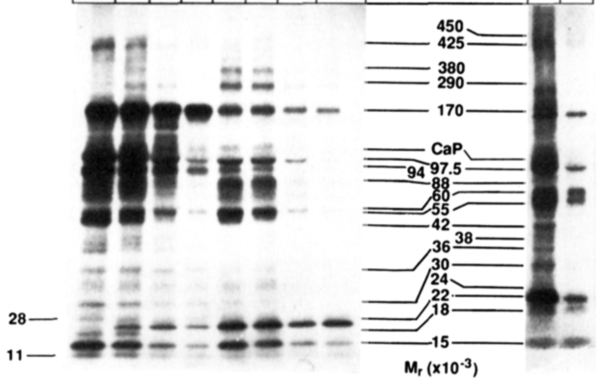


FIGURE 7: Effect of exogenous phosphatases on JTC (lanes 1–4, 9, and 10) and light SR (lanes 5–8) phosphoproteins, as revealed by autoradiography. The initial phosphorylation was carried out as described under Materials and Methods, with 200 μM [γ-^{32}P]ATP for 1 min, in the presence (E+) or absence of 1 mM EGTA and no added calcium (E–), or of 100 μM free Ca^{2+}. For the samples in lanes 2–4, 6–8, and 9–10 (+), a chase with nonradioactive ATP was then started as described for Figure 6, and the reaction was allowed to proceed for 20 min. For the samples in lanes 3 and 4 and 7 and 8, the chase medium (see Materials and Methods) included 1 mM EGTA and 59 units/mL phosphatase 1 or 70 units/mL catalytic subunit of phosphatase 2A as indicated in the figure. For the samples in lanes 9 and 10, the chase medium included 100 μM free Ca^{2+} (1.126 mM $CaCl_2$ + 1 mM Tris–EGTA), and for sample 10, it was supplemented with 1.7 mg of calcineurin (CN)/mL. For the control samples in lanes 1 and 5, the phosphorylation reaction was stopped before the chase with nonradioactive ATP. This experiment shows that the phosphoprotein turnover is markedly enhanced by the addition of exogenous phosphatases.

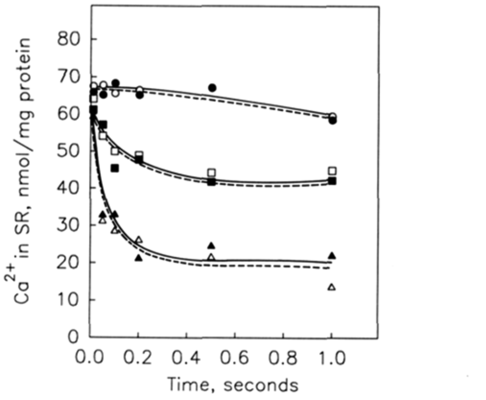


FIGURE 8: Ca^{2+} release from JTC vesicles loaded actively by utilization of ATP or acetyl phosphate. Samples of JTC vesicles were loaded actively for 2 min in the presence of ATP (solid symbols) or acetyl phosphate (open symbols) and placed on a rapid filtration apparatus (Materials and Methods). The loaded vesicles were then flushed either with nonrelease medium (○, ●) or with media containing 5 μM free Ca^{2+} (□, ■) or 5 μM free Ca^{2+} + 1 mM ATP (△, ▲).

activity associated prevalently with the JTC. In the absence of Ca^{2+}, 170 000-, 94 000-, 60 000-, and 55 000-Da protein components are more distinctly phosphorylated (Figures 1 and 2). The 170 000-Da protein reacts with Stains-all as a glycoprotein, binds doxorubicin (Zorzato & Volpe, 1988), and has been suggested to be the caffeine receptor of the SR Ca^{2+} release channel (Rubstov & Murphy, 1988). It should be pointed out that the 450 000-Da channel protein is highly susceptible to proteolysis (Seiler et al., 1984; Chu et al., 1988b; Lai et al., 1988; Trimm et al., 1988; Meissner et al., 1989) and the 170 000-Da band may contain such a derived frag-

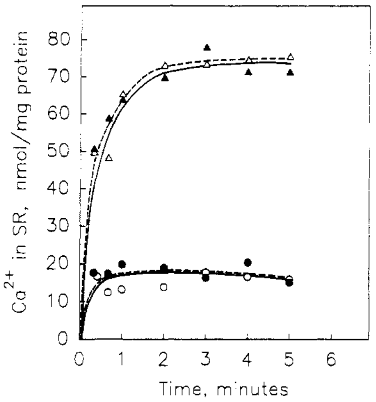


FIGURE 9: Effect of calmodulin on active Ca^{2+} transport by JTC. Active Ca^{2+} uptake was started by addition of 1 mM ATP to the reaction medium (see Materials and Methods) in the presence (solid symbols) or in the absence (open symbols) of 34 μg (~2 μM) of calmodulin/mL. The Mg^{2+} concentration in the loading medium was varied from 1 (○, ●) to 10 (△, ▲) mM to keep the ryanodine release channel open or closed, respectively.

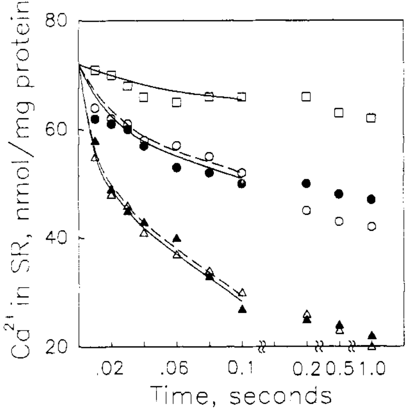


FIGURE 10: Calmodulin-dependent protein phosphorylation does not alter the kinetics of Ca^{2+}- and nucleotide-induced Ca^{2+} release from JTC. Ca^{2+} release was measured after loading JTC with 1 mM ATP for 2 min as described above, in the absence (closed symbols) or in the presence (open symbols) of calmodulin (34 μg/mL). The loaded vesicles were then placed on filters in a rapid filtration apparatus and exposed for electronically controlled times to release media containing 5 μM free Ca^{2+} (○, ●), or 5 μM free Ca^{2+} + 1 mM AMP-PNP (△, ▲), or nonrelease medium (□) as described in Materials and Methods.

ment. Furthermore, the dihydropyridine receptor has a 170000-Da component that can be phosphorylated (Curtis & Catterall, 1985; Imagawa et al., 1987a), and the JTC microsomal fraction is likely to contain T-tubule membrane contaminants (our JTC preparations display 8% [^{3}H]-PN200-11 binding capacity as compared to T-tubule preparations).

A 97500-dalton (likely phosphorylase) and a 55000–60000-Da doublet (Figures 1 and 2) were also phosphorylated more heavily in the presence of micromolar Ca^{2+}. The latter doublet includes calmodulin-dependent protein kinase subunits (Stull et al., 1986; Cohen, 1988). In fact, the 60000-Da protein of SR has been reported to bind calmodulin (Vale, 1988) and to undergo autophosphorylation (Tuana & MacLennan, 1988).

The large increase of protein phosphorylation following addition of calmodulin (Figure 3) demonstrates the prominent association of calmodulin-dependent protein kinase and related substrates with the JTC. This association is not interfered with by high ionic strength washings carried out during purification of the JTC subfraction. Its specificity is underlined by the magnitude of the calmodulin effect on JTC (Figures 3 and 5), the lower effect of calmodulin on light SR, and the low levels of cyclic AMP dependent protein kinase activity in both JTC and light SR (Figure 6). With respect to T-tubule membranes, it is noteworthy that purified triad preparations exhibit similar overall phosphorylation levels under conditions of intrinsic kinase, calmodulin-dependent kinase, or cAMP-dependent kinase activation (Imagawa et al., 1987a). On the contrary, JTC vesicles display approximately 10-fold higher phosphorylation levels (Figure 3) under conditions of calmodulin activation.

The relatively large number of JTC protein components (Figure 3) phosphorylated by the membrane-bound, calmodulin-dependent kinase is consistent with a multifunctional (Stull et al., 1986; Cohen, 1988) character of this enzyme. Some of the phosphorylated proteins are likely to be enzymes involved in glycogen metabolism (Entman et al., 1980; Wanson & Drochman, 1972; Goldstein et al., 1985); Villa-Moruzzi, 1986) such as the 140000-, 128000-, 42000-, and 17000-Da subunits of phosphorylase *b* kinase, the 160000-Da glycogen debranching enzyme, the 97500-Da phosphorylase, and the 88000-Da glycogen synthase (Campbell & Shamoo, 1980; Varsanyi & Heilmeyer, 1981; Jennissen & Lahr, 1980). Phosphorylation involves also the 450000-Da ryanodine receptor and its large proteolytic fragments (Imagawa et al., 1987b; Lai et al., 1988; Inui et al., 1987; Meissner et al., 1989), the 170000-Da glycoprotein, the 106000-Da Ca^{2+}-ATPase, and the 55000–60000-Da doublet including the calmodulin binding kinase subunit and the protein reported by Kim and Ikemoto (1986). It is of interest that millimolar Ca^{2+} inhibits calmodulin-dependent phosphorylation of JTC proteins, with the exception of the 60000-Da protein (Figure 3). The very large difference in the effects of low and high [Ca^{2+}] must be kept in mind when considering whether the calmodulin-dependent phosphorylation affects selectively the 60000-Da protein in physiological conditions. It is noteworthy that Plank et al. (1988) did not find a satisfactory correlation between phosphorylation of this protein and Ca^{2+}-induced Ca^{2+} release.

Although some of the JTC proteins may be phosphorylated without functional consequence, the specific association of high calmodulin-dependent kinase with JTC suggests that this enzyme is involved in regulation of functions linked to the excitation–contraction coupling. Therefore, we investigated a possible regulation of the Ca^{2+}-induced Ca^{2+} release, which is the specific function exhibited by the isolated cisternal membranes (JTC), through the high conductance channel of the ryanodine receptor protein (Yamamoto & Kasai, 1982; Meissner et al., 1986; Smith et al., 1985). As shown in Figures 8–10, we found no significant change of this function that could be related to protein kinase activation. It should be pointed out that an inhibitory effect of calmodulin on the kinetics of calcium efflux was demonstrated by Meissner (1986). This effect, however, was not produced through phosphorylation, since the vesicles were not exposed to ATP at any time during the experimentation [see also Smith et al. (1989) and Plank et al. (1988)].

As for our inability to observe a functional effect of protein phosphorylation on the efflux kinetics, we consider that protein activation may affect functions which are not tested by experiments on Ca^{2+}-induced Ca^{2+} release from isolated JTC vesicles but are parts of the excitation–contraction coupling mechanism in the fiber. In fact, there are reasons to believe that influx of extracellular calcium is not necessarily required for excitation–contraction coupling in the muscle fiber (Rios & Brum, 1987; Brum et al., 1988). Rather, the ryanodine receptor channel of junctional SR may be coupled to the dihydropyridine receptor of the T tubular membrane through a voltage-sensitive step (Tanabe et al., 1988; Block et al., 1988). It is of interest that a high level of multifocal calcium/calmodulin dependent protein kinase is found in neuronal tissue and synaptic vesicles, possibly related to modulation of neuronal functions and synaptic coupling (Schulman, 1988). The importance of our present findings lies in the identification of the SR fraction (i.e., junctional-cisternal SR) that undergoes high levels of phosphorylation, the specific type of kinase (i.e., calmodulin-dependent protein kinase) which is prominently associated with this membrane fraction, and the optimal conditions that are required for phosphorylation and dephosphorylation.

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