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; Hossy et al., 1986; Imagawa et al., 1987a; Catterall et al., 1988), and neuronal signal transduction (Hemmings et al., 1989). 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 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.

**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 55000-60000-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 (~45000 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.

**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), tetrathiomolybdate 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 tetrathiourea salt of adenosine 5′-O-(3-thiotriphosphate) (ATP-γS) 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 hindleg and backs of New Zealand White rabbits were used for the light and junctional sarcoplasmic reticulum preparation [R2 fraction and R4 fraction (Chu et al., 1988)].
CaCl

ATP [specific activity (-3-7)

γ

(pH 7), 0.2-0.5 mM [Liquots (200

added to the mixture at 23-25 °C to start the reaction. Al-

described above. Either Ca

SR (200

% SDS, 1.25% 2-mercaptoethanol, 0.312 mM Tris-HCl (pH

so that the final mixture contained 0.025% bromphenol blue,

adding concentrated denaturing buffer (for gel electrophoresis),

containing 80-150

morpholino)propanesulfonic acid) (pH 7)], 10 mM MgCl

Reticulum.

The reaction medium for phosphorylation of SR

was 5 mM MgCl

specific activity ~9000-15000 cpm/nmol). For active loading, 50-

μg aliquots of SR protein were allowed

with 1 mL of nonrelease medium, and release of intravesicular

was then started by perfusing the vesicles on the filter

figure legends. For protein identification, immunoblot strips

were prepared by using light SR, triads, and JTC phosphory-

lated with Ca

and Ca

/20 mM Tris-MOPS (pH 7) and then processed for liquid scintillation counting.

32

Ca2+

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 

32
calcineurin was used, CaCl

was added to maintain the

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
cAcCl

, 10 mM MgCl

, 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
cAcCl

and buffered salt, 50-μg aliquots were diluted with 1 mL of nonrelease medium (containing 10 mM MgCl

and no Ca

) 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 

32
calcineurin was then started by perfusing the vesicles on the filter with a medium containing 5 μM free Ca

and buffered salt, in the presence and absence of 1 mM Na

ATP or AMP-PNP. The nonrelease medium containing 10 mM MgCl

and no Ca

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

, 80 mM KCl, 20 mM Tris-MOPS (pH 7), 0.2-0.5 mM [γ-32P]ATP [specific activity (-3-7) × 10

, 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

-independent protein phosphatase or calceinurin was then added in a concentrated dephospho-

mM Na

ATP, 5 mM MgCl

, 1 mM MnCl

, buffered salt, and 1 mM Tris-EGTA. When calceinurin was used, CaCl

was added to maintain the desired free [Ca

]. 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-32P]ATP, and the reaction was stopped at the appropriate time interval by 1 mL of ice-cold 10% trichloroacetic acid/0.1 mM NaH

PO

/L mM Na

ATP. 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 Na

PO

/L mM Na

ATP) 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 (205000), β-galactosidase (116300), phosphorylase b (97 500), bovine albumin (68 000), ovalbumin (43000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (30000), 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

and Ca

/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

 concentrations were estimated according to Fabiato and Fabiato (1979).

RESULTS

Subtractions 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

/ATPase, which is the enzyme responsible for active transport of Ca

, is a prominent component of both preparations (Figure 1A). On the other hand, a prominent

et al., 1988a), respectively]. In some preparations, proteolytic inhibitors were added to all media, as follows: 77 mM aproti-

, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.7 μM pepstatin A, and 0.23 mM phenylmethylsulfonyl fluoride (PMSF). Triad preparations were obtained by a modification of the method of Mitchell et al. (1983), as described by Sharp et al. (1987).

4

Ca2+

Uptake. Active Ca

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

, and 100 μM 45CaCl

(specific activity ~9000 cpm/nmol). Either 1 mM Na

ATP 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

/80 mM KCl/20 mM Tris-MOPS (pH 7) and then processed for liquid scintillation counting.

32

Ca2+

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
calcineurin was then started by perfusing the vesicles on the filter with a medium containing 5 μM free Ca

and buffered salt, in the presence and absence of 1 mM Na

ATP or AMP-PNP. The nonrelease medium containing 10 mM MgCl

and no Ca

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.
calsequestrin band and a large protein (450000 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 170000-Da protein binds doxorubicin and has been shown to react with Stains-all, which is indicative of its glycoprotein character (Zorzato & Voipe, 1988). On the other hand, as the preparation inevitably contains some transverse tubule membrane contaminants, the 170000-Da and 52000-Da components may be subunits of the dihydropyridine receptor (Imagawa et al., 1987a). It is noteworthy that the 425 000-Da and the 170000-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$^{-6}$ M) and no added protein kinase, several JTC bands appeared phosphorylated, corresponding to 170000-, 94000-, 60000-, and 55 000-Da proteins. Some phosphorylation was also observed 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 170000-, 94000-, and 55 000-Da proteins was reduced, while the low level phosphorylation of 97 500- (likely glycogen phosphorylase), 88000-, 60000-, 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 60000-, 97000-, and 22000-Da proteins. On the other hand, Ca$^{2+}$ inhibits phosphorylation of the 94000- 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$^{-5}$ M range. Under optimal conditions, numerous proteins of JTC were phosphorylated (Figure 3), including the 450 000-Da ryanodine receptor and the 425000-, 88000-, 60000-, 55000-, 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 <11000 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-
concentration: 1 mM EGTA

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 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, partial loss 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 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 425000-, 390000-, 280000-, 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 to the incubation mixture, 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...
Phosphorylation of Junctional Proteins

**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 $[^{32}P]$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 ATP-yS (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 subtractions of SR vesicles revealed protein kinase activity associated prevalently with the JTC. In the absence of Ca$^{2+}$, 170000-, 94000-, 60000-, and 55000-Da protein components are more distinctly phosphorylated (Figures 1 and 2). The 170000-Da protein reacts with Stains-all as a glycoprotein, binds doxorubicin (Zorzato & Voipe, 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 170000-Da band may contain such a derived frag-
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 subtraction. 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; Hanson & 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 97 500-Da phosphorylase, and the 88000-Da glycogen synthase (Campbell & Shamo, 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 170 000-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 Kiri 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 functional SR may be coupled to the dihydropyridine receptor of the T tubular membrane through...
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