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Role of Calmodulin in Skeletal Muscle Sarcoplasmic Reticulum†

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ABSTRACT: Three proteins having M_r of 20 000, 35 000, and 57 000 were phosphorylated by a calmodulin-dependent system in fast skeletal muscle sarcoplasmic reticulum (SR). The 20 000-dalton phosphoprotein was an acidic proteolipid distinct from phospholamban, which was present in cardiac SR preparations. The 57 000-dalton phosphoprotein became phosphorylated very rapidly ($t_{1/2}$ = 5–10 s at 0 °C) and was distinct from calsequestrin and the 53 000-dalton glycoprotein, as judged from the electrophoretic mobility on neutral Laemmli gels and from its detergent-extraction characteristics. None of the three phosphoproteins interacted directly with calmodulin, implying that they were not the regulatory subunit of the calmodulin-dependent kinase. The calmodulin-dependent kinase(s) responsible for the phosphorylation of the three protein substrates was (were) membrane bound. Its K_m(ATP) was about 200 μM, and at the probable physiological calmodulin concentration of 1–2 μM, its K_m(Ca) was 0.7 μM. The 57 000-dalton phosphoprotein was dephosphorylated by an endogenous phosphatase activity, which was also activated by the Ca–calmodulin complex.

The role of calmodulin in heart sarcoplasmic reticulum (SR) has been intensively investigated in the last 3 or 4 years. A stimulation of Ca^{2+} transport in cardiac SR vesicles was first reported by Katz & Remtulla (1978) and soon confirmed in several other laboratories (LePeuch et al., 1979; Wuytack et al., 1980; Bilezikjian et al., 1980). As a result of these studies, a complex calmodulin-dependent system for the regulation of the cardiac SR Ca^{2+} pump has now come to light. It apparently operates in parallel with the regulatory system, which depends on cAMP and a specific kinase and which phosphorylates the hydrophobic intrinsic protein phospholamban (Tada et al., 1975; Wray & Gray, 1977). The calmodulin-dependent system requires Ca^{2+}, is mediated by a specific kinase (LePeuch et al., 1979), and also phosphorylates phospholamban. The sites of phospholamban phosphorylation by the two systems are apparently different (LePeuch et al., 1979). Direct evidence for the existence of a calmodulin-dependent protein kinase in heart SR has recently been provided by Jones & Wegener (1981). A protein fraction containing kinase activity was separated from detergent-solubilized heart SR by calmodulin affinity chromatography and shown to phosphorylate more than one protein in the SR membrane.

Calmodulin is now known to be present in SR different from heart (Carafoli et al., 1980), including skeletal muscle. Two detailed studies on the latter tissue have recently appeared (Chiesi & Carafoli, 1982; Campbell & MacLennan, 1982). In one (Campbell & MacLennan, 1982), calmodulin was shown to mediate the phosphorylation of two SR proteins, a major one having M_r of 60 000 and a minor one of M_r 20 000. The suggestion was made that the calmodulin-dependent phophorylation may mediate the release of Ca^{2+} from SR vesicles. In the other (Chiesi & Carafoli, 1982), calmodulin was found to promote the phosphorylation of three SR proteins, having M_r of 57 000, 35 000, and 20 000. Also in this study, the conclusion was reached that the phosphorylation of these three proteins has no influence on the active Ca^{2+} uptake but may regulate its release from the vesicles.

The purpose of the present work has been the systematic study of the calmodulin-dependent phosphorylation of proteins in the various cell fractions leading to the final isolation of purified skeletal muscle SR membranes and the characterization of those unequivocally attributable to the SR membrane in terms of their possible role in the calmodulin regulation system. The results have corroborated the previous conclusion that skeletal muscle membranes contain three proteins that are substrates of the calmodulin-promoted phosphorylation. Those having M_r of 57 000 and 20 000 appear to be the major substrates. None of the three proteins, however, interacts directly with calmodulin; i.e., they are not identical with the calmodulin-dependent kinase nor with its regulatory subunit. The study has shown that, in addition to the specific calmodulin-dependent kinase, skeletal muscle SR membranes also contain a calmodulin- (plus Ca^{2+}) dependent phosphatase. The phosphorylated M_r 57 000 protein is one of its substrates.

Materials and Methods

Hexokinase (type VII) and cAMP-dependent protein kinase from rabbit muscle were obtained from Sigma. [γ-^{32}P]ATP (2–10 Ci/mmol) was obtained from New England Nuclear. Calmodulin was isolated from bovine brain according to Watterson et al. (1976). Azidocalmodulin was prepared as described (Andreasen et al., 1981). Calmodulin was conjugated to CNBr-activated Sepharose 4B (Pharmacia) as previously described (Niggli et al., 1979). SR was isolated from rabbit white muscles according to Eletr & Inesi (1972).

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Kinase Assay. Unless otherwise stated, the activity of endogenous calmodulin-dependent kinase was studied at room temperature in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Mops,[1] pH 7, and 5 μM ionophore A23187 ("basic medium"). The desired free Ca concentration was obtained with a Ca–EGTA buffering system. Free Ca was calculated with a computer program (Fabiato & Fabiato, 1979) taking into account binding to EGTA and ATP and competition with other cations. SR vesicles (or other fractions) were incubated in the basic medium in the presence, when required, of various amounts of calmodulin. The reaction was started by the addition of the desired [γ-^{32}P]ATP concentration (specific radioactivity 0.2 Ci/mmol) and quenched after sequential time intervals with $NaDodSO_4$ solubilizing buffer (final concentration was 2% $NaDodSO_4$, 10% glycerol, 2 mM dithiothreitol, 0.5 mg of bromphenol blue/mL, and 50 mM sodium phosphate buffer, pH 7).

The endogenous calmodulin-dependent kinase activity could be stimulated also by an azidocalmodulin derivative. In these experiments, SR vesicles (1 mg/mL) were incubated in the presence of Ca and azidocalmodulin (1 μM). The mixture was illuminated for 30 s at 350 nm and then washed in EGTA buffer to extract unbound azidocalmodulin. The pelleted vesicles were then phosphorylated as described above. The effect of cAMP-dependent kinase was studied by preincubating SR vesicles in the basic medium supplemented with 1 μM cAMP and 0.1 mg of cAMP-dependent protein kinase/mL.

Phosphatase Assay 1. Endogenous phosphatase activity was studied by following the dephosphorylation kinetics of the endogenous SR proteins, which were previously phosphorylated in a Ca–calmodulin-dependent way. The phosphatase reaction was studied after all residual [γ-^{32}P]ATP was rapidly consumed by excess hexokinase. In detail, the experiment was as follows: SR vesicles (1 mg/mL) were phosphorylated in the basic medium containing 100 mM glucose, 1 μM calmodulin, 0.7 mM $CaCl_2$, 1 mM EGTA, and 200 μM [γ-^{32}P]ATP. After 20 s, hexokinase was rapidly added to the reaction mixture (100 units/mL), and the dephosphorylation rate of endogenous phosphoproteins was studied by quenching aliquots of the suspension at sequential time intervals. When required, the dephosphorylation medium was modified by making appropriate addition 5 s after hexokinase.

The rate of ATP consumption by the hexokinase was investigated in control experiments by measuring the Ca-uptake activity of SR vesicles. These experiments were carried out under similar conditions; however, A23187 was omitted and 20 μM Ca was added instead of the Ca–EGTA buffering system. Ca uptake was followed with a multichannel air-turbin spectrophotometer (University of Pennsylvania, Biomedical Instrumentation Group) and with 150 μM metallochromic indicator murexide. These experiments showed that the Ca-uptake reaction of SR vesicles was completely blocked within 5 s after hexokinase addition.

Phosphatase Assay 2. Also in these experiments, the endogenous SR phosphoproteins were first labeled with [γ-^{32}P]ATP as described under Phosphatase Assay 1. After 10 s, the endogenous phosphatase activity was inhibited by diluting the mixture into ice-cold basic medium containing 1 mM unlabeled ATP and 10 mM EDTA. The vesicles were pelleted by centrifugation for 20 min at 140000g and resuspended in ice-cold basic medium containing 1 mM EGTA. The washing procedure removed most (>95%) of the exogenous calmodulin, added to phosphorylate the vesicles (as judged by gel electrophoresis). The phosphatase activity was considerably inhibited during the washing step so that a considerable amount of phosphoprotein (20–40% of the maximal level) was conserved. Dephosphorylation rates of these residual phosphoproteins were immediately studied by diluting the SR vesicles at room temperature into basic medium supplemented either with (a) 1 mM EGTA, (b) 1 mM EGTA and 0.7 mM $CaCl_2$ (free Ca = 1.1 μM), or (c) 1 mM EGTA, 0.7 mM $CaCl_2$, and 2 μM calmodulin.

Extraction of Proteolipids from SR Vesicles. An 80-μL aliquot of SR vesicles (1 mg/mL) was phosphorylated in the presence of [γ-^{32}P]ATP, 2 μM calmodulin, and 1.1 μM free Ca. The reaction was quenched after 30 s by rapid mixing with 1690 μL of an organic phase consisting of chloroform–methanol (13:5) and 7 μL of 6 M HCl. A 25-μL aliquot of unphosphorylated SR vesicles (5 mg/mL) was added as carrier. After vortexing for 5 min, the mixture was centrifuged for 10 min at 10000g to separate the organic from the small aqueous phase. Most of the heavier organic phase was carefully removed without disturbing the protein fraction concentrated at the interface between the two phases. The organic solvent was then evaporated under a N_2 stream, and the compositions of the residual protein and of the extract were investigated by electrophoresis and autoradiography.

Gel Electrophoresis and Autoradiography. The protein composition of SR vesicles was investigated by polyacrylamide gel electrophoresis according to Laemmli (1970) with minor modifications. The separating gel contained 10% acrylamide. When specified in the text, the pH of the separating gel was adjusted to 6.8 instead of 8.8. Under such conditions, it was possible to greatly expand the molecular weight region between 35000 and 100000, maintaining good resolution. After electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue, destained, and dried. Thereafter, radioactive gels were autoradiographed at −70 °C for 2–5 days with X-ray-sensitive films and an intensifying screen (Kyokko H5). The phosphoproteins detected according to this procedure were hydroxylamine resistant. The relative level of radioactivity associated with the proteins was determined either with densitometry scanning of the autoradiography plates or by cutting the radioactive protein bands and counting the radioactivity in a scintillation cocktail. Protein concentration was measured according to Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Calmodulin-Dependent Phosphorylation of Proteins in Skeletal Muscle Homogenate and Other Fractions Obtained during Purification of SR. The phosphorylation experiments to be described have been performed under three sets of experimental conditions: (a) excess EGTA, (b) Ca^{2+}, and (c) Ca^{2+} and calmodulin. In all cases, the concentration of ATP was 200 μM [the K_m(ATP) of most calmodulin-dependent kinases lies in the vicinity of 200 μM (Walsh et al., 1979)], and a standard reaction time of 10 s was used. Figure 1 shows that under these experimental conditions six major proteins of the homogenate became labeled. The most important, having an M_r of 70000, was not influenced by calmodulin and probably belongs to the soluble phase of the muscle cell, since it became enriched in the supernatant obtained from the centrifugation of the SR membranes (see Figure 1, fractions III and V) and in the light SR fraction (see Figure 1, LSR), which is contaminated by soluble proteins. Another protein, having an M_r of 15000, became phosphorylated in a Ca^{2+}-

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; TFP, trifluoperazine; Mops, 4-morpholinepropanesulfonic acid; DOC, deoxycholate; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

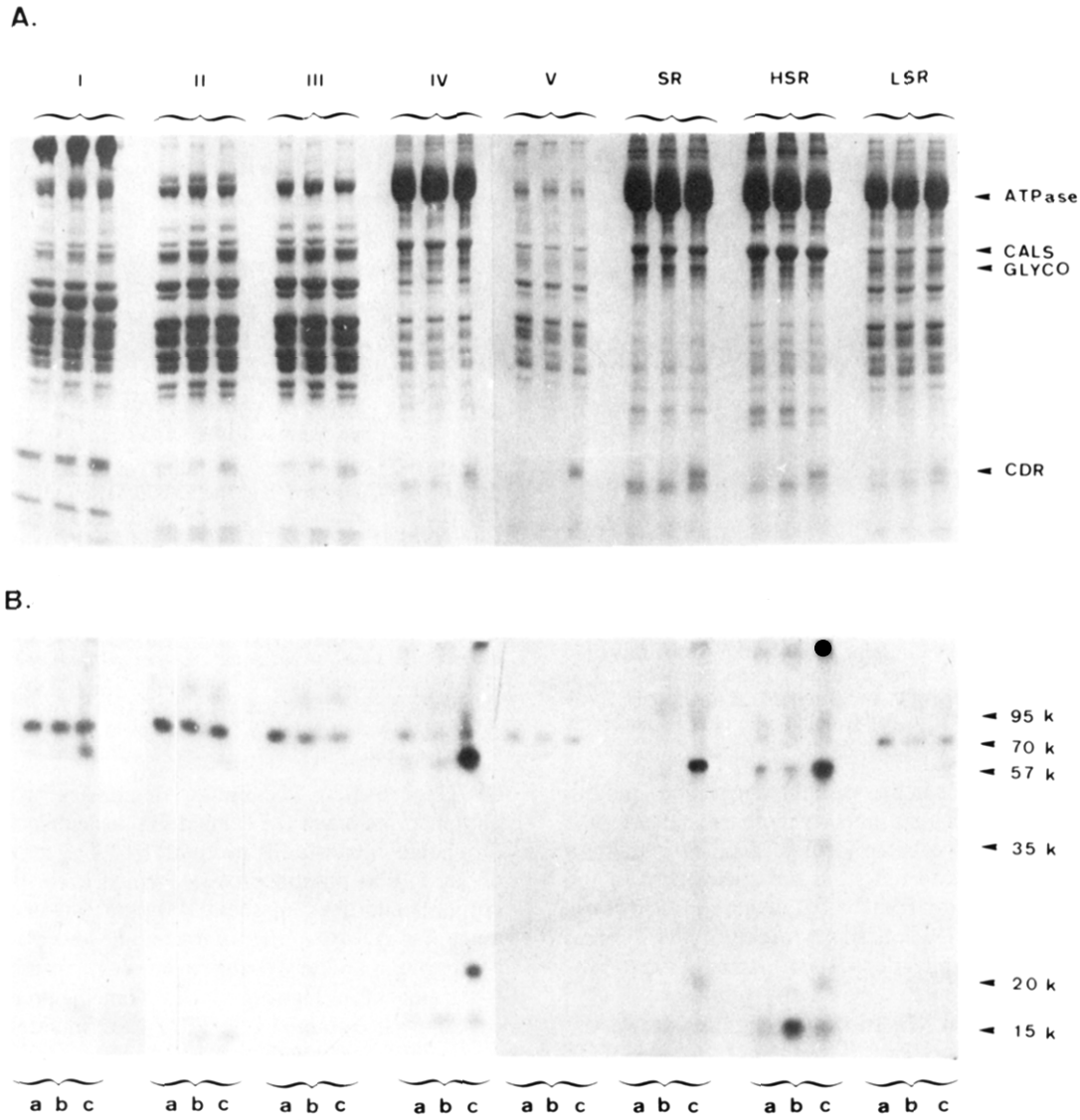

FIGURE 1: Distribution of calmodulin-dependent phosphorylating activity in various fractions derived from fast skeletal muscle. SR vesicles were isolated from fast skeletal muscle as described (Eletr & Inesi, 1972). Fraction I is the total muscle homogenate. Fraction II is the supernatant after low-speed centrifugation of the homogenate. Fraction III is the supernatant after sedimentation of the crude SR preparation (Fraction IV) at 42000*g* for 90 min. Fraction IV was incubated in 0.6 M KCl and after a 20-min centrifugation at 14000*g*, four membraneous fractions were separated: a light fluffy fraction on top of the centrifuge tube (LSR), a broad intermediate fraction (SR), a heavy fraction near the bottom of the tube (HSR), and a pellet that was discarded. The intermediate SR fraction was further centrifuged and separated from the soluble proteins present in the supernatant (fraction V). The calmodulin-dependent phosphorylation of muscle proteins was studied on the different fractions described above by incubating 1 mg of protein/mL at room temperature in the presence of 200 μM [γ-^{32}P]ATP. The phosphorylation reactions were stopped after 10 s, and the quenched protein samples were separated electrophoretically and autoradiographed as described under Materials and Methods. For each fraction tested, phosphorylation was carried out in the presence of (a) EGTA, (b) 0.7 μM free Ca, or (c) 0.7 μM free Ca and 10 μg of calmodulin/mL. (A) Protein staining of the various fractions: ATPase = Ca–Mg ATPase of SR vesicles; CALS = calsequestrin; GLYCO = 53000-dalton glycoprotein; CDR = calmodulin. (B) Autoradiography of the gel presented in panel A.

dependent but calmodulin-independent way. This phosphoprotein is probably membrane bound, since it became enriched in the fraction containing SR (fractions IV, SR, and HSR in Figure 1). The phosphorylation of a third protein, having an M_r of 95000, required Ca^{2+} but not calmodulin. This phosphoprotein was enriched in the fraction containing soluble proteins (fraction III in Figure 1) and was probably identical with phosphorylase, since it comigrated with it.

The three other phosphoproteins were those identified previously (Chiesi & Carafoli, 1982). They had an absolute requirement for Ca^{2+} and added calmodulin and clearly belong to the SR membranes, since they became very evidently enriched in the fractions containing SR (fractions IV, SR, and HSR in Figure 1). Although the M_r of the three phosphoproteins are given here as 57000, 35000, and 20000, on some particularly well-resolved autoradiography gels it was possible to see that the bands corresponding to the three proteins actually consisted of doublets separated by a 1000–2000-dalton difference.

The phosphoproteins of M_r 57000, 35000, and 20000 are the three major targets of the calmodulin-dependent (kinase) activity of SR membranes, and it seems logical to postulate that their phosphorylation may be relevant to the function of the organelle. So far, however, no correlation has been found between the very evident stimulation of these three proteins by calmodulin and the active uptake of Ca^{2+} by SR. An alternative possibility would be the regulation of the release, rather than the uptake, of Ca^{2+} by the calmodulin-promoted phosphorylation of the three proteins. The finding that their level of phosphorylation was higher in SR and HSR than in

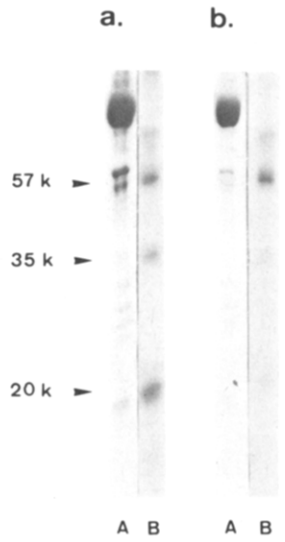

FIGURE 2: Effect of Lubrol extraction on phosphorylation of the 57000-dalton phosphoprotein. SR vesicles (0.5 mg/mL) were partially solubilized with 0.1% Lubrol in a medium containing 500 mM sucrose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Mops, pH 7.0, and 2 mM dithiothreitol. Solubilized material was removed by centrifugation. Phosphorylation was carried out as usual in the presence of Ca, calmodulin, 200 μM [γ-^{32}P]ATP, and 1 mg/mL of untreated SR (a) or extracted SR vesicles (b): (A) protein staining; (B) autoradiography.

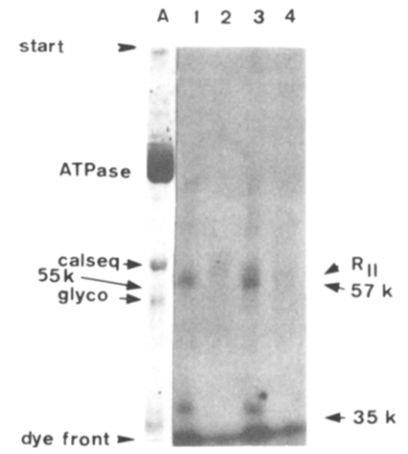

FIGURE 3: Electrophoretic separations of calsequestrin, the 57000-dalton phosphoprotein, and the 53000-dalton glycoprotein. Phosphorylation reactions were carried out for 10 s at room temperature with 200 μM [γ-^{32}P]ATP and 20 μM $CaCl_2$ as described under Materials and Methods. The reaction mixtures contained: (1) 1 mg of SR/mL and 10 μg of calmodulin/mL; (2) 1 mg of SR/mL, 10 μg of cAMP-dependent protein kinase/mL, 1 mM EGTA, and 1 μM cAMP; (3) 1 mg of SR/mL, 10 μg of cAMP-dependent protein kinase/mL, 1 μM cAMP, and 10 μg of calmodulin/mL; (4) 10 μg of cAMP-dependent protein kinase/mL and 1 μM cAMP. Quenched samples were electrophoretically separated on a 10% Laemmli gel system but with a pH of 6.8 instead of the usual pH of 8.8 in the separating gel.

LSR (Figure 1) would be in line with this suggestion. Indeed, SR and HSR contain a larger proportion of terminal cisternae (Meissner, 1975), the probable locus of Ca^{2+} release, than LSR. This could be inferred from the enrichment in the "marker" calsequestrin in SR and HSR (Figure 1). However, the possibility that LSR contains a highly active protein phosphatase activity, which dephosphorylates the three proteins, cannot be ruled out.

At the moment, then, the functional significance of the calmodulin-promoted phosphorylation of the M_r 57000, 35000, and 20000 proteins remains obscure and will probably be clarified only when suitable conditions will be found for the study of the effects of calmodulin on the release of Ca^{2+} from SR. This may be a long way off. Meanwhile, information on the targets of calmodulin activity in skeletal muscle SR and on their interaction with the activator may significantly advance knowledge in this area.

In the following sections, the two major substrates of the calmodulin-directed phosphorylation, present in the SR fraction, the M_r 57000 and 20000 proteins, will be characterized. The quantitatively minor M_r 35000 component has not been considered in the present study.

M_r 57000 Phosphoprotein. The region in Laemmli polyacrylamide gels where this protein is found contains at least three well-known SR proteins: calsequestrin (M_r 63000), the high-affinity Ca^{2+}-binding protein (M_r 55000), and the M_r 53000 glycoprotein. Other minor components may also be present, like the regulatory subunit of the cAMP-dependent protein kinase (R_{II}). The assignment of the radioactivity seen in the autoradiographs in this region of the gels to a specific protein component was therefore not obvious. The following observations, however, ruled out calsequestrin and the glycoprotein as possible substrates of the calmodulin-dependent protein kinase: (a) Extraction of SR membranes with the detergent Lubrol released most of the glycoprotein and calsequestrin to the medium. The M_r 57000 phosphoprotein, on the other hand, remained associated with SR and, most interestingly, could still be phosphorylated in the presence of calmodulin to a degree comparable to that of the unextracted SR (Figure 2). The finding demonstrates that both the phosphoprotein and the calmodulin-dependent kinase are hydrophobic, intrinsic SR proteins. (b) The maximal amount of M_r 57000 phosphoprotein formed corresponded, under optimal conditions, to about 100 pmol/mg of protein. This amount is at least 1 order of magnitude lower than that of the glycoprotein and of calsequestrin. It corresponds, however, to the amounts of high-affinity Ca^{2+}-binding protein (as judged by Coomassie stained gels). (c) Careful side-by-side comparison of Coomassie Blue stained and autoradiographed gels show that the M_r 57000 phosphoprotein migrated to a position that was intermediate between that of a calsequestrin and that of the glycoprotein. This was particularly evident in neutral Laemmli gels (see Materials and Methods), in which the M_r region between 30000 and 100000 was greatly expanded. In Figure 3, it is clearly seen that the phosphoprotein migrated close to the high-affinity Ca^{2+}-binding protein (M_r 55000).

Lane 2 of Figure 3 shows that the addition of cAMP plus exogenous cAMP-dependent protein kinase resulted in the phosphorylation of a faint and diffuse band in the M_r region between 58000 and 60000. This faint radioactive band, however, was visible also in the control sample without SR membranes (lane 4 of Figure 3), and it is therefore very probable that it corresponded to the autophosphorylated regulatory subunit of the added protein kinase (R_{II}). Figure 3 also shows (compare lanes 1 and 3) that the extent of incorporation of ^{32}P was higher in the presence of *both* cAMP and calmodulin than in the presence of calmodulin alone. The cAMP-induced stimulation of the incorporation of radioactivity into the calmodulin-sensitive protein was evident. A quantitative estimate, however, was prevented by the presence of the overlapping autophosphorylated R_{II} subunit of the cAMP-dependent kinase.

In conclusion, then, the M_r 57000 phosphoprotein is distinct from calsequestrin, the glycoprotein, and the (contaminating) R_{II} subunit of the cAMP-dependent protein kinase. It may be identical with the high-affinity Ca^{2+}-binding protein or, more probably, it may correspond to a hitherto unidentified intrinsic protein component of the SR membrane.

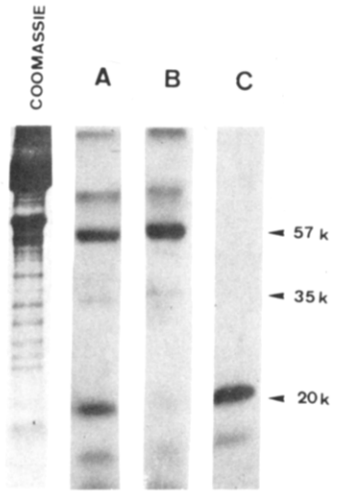

FIGURE 4: Extraction of the 20 000-dalton phosphoprotein from SR vesicles. SR vesicles were phosphorylated with [γ-^{32}P]ATP in the presence of calmodulin and Ca as described in Figure 3. The reaction was quenched, and the vesicles were extracted with an acidic chloroform–methanol mixture as described under Materials and Methods. The protein content of extracted vesicles and solubilized material was checked by using Laemmli gel electrophoresis and autoradiography of the gel (A–C). (A) Autoradiogram obtained with untreated SR vesicles (the protein staining of this fraction is shown on the left). (B) Autoradiogram obtained with chloroform–methanol-extracted vesicles. (C) Autoradiogram of the solubilized material.

M_r 20 000 Phosphoprotein. The apparent M_r of the second major phosphoprotein product of calmodulin activity in skeletal muscle SR is close enough to that of cardiac phospholamban (22 000) to warrant a comparative investigation of the properties of the two proteins. Phospholamban is an acidic proteolipid (LePeuch et al., 1980) that is contained in cardiac SR membranes in a 1:1 molar ratio of the Ca-pumping ATPase. It regulates (i.e., stimulates) the Ca^{2+}-accumulating activity of cardiac SR by interacting in a still unclarified way with the ATPase, after becoming phosphorylated by independent cAMP and calmodulin-sensitive kinases.

The M_r 20 000 phosphoprotein of skeletal muscle is apparently also an acidic proteolipid, since it was quantitatively extracted into the organic phase by treating the vesicles with an acidic methanol–chloroform mixture (Figure 4) (under the same conditions, the previously mentioned Ca^{2+}-dependent but calmodulin-independent M_r 15 000 phosphoprotein was also quantitatively extracted into the organic phase, indicating that it may also be an acidic proteolipid). The M_r 20 000 protein, however, after phosphorylation to maximal levels in the presence of calmodulin, represented only between 50 and 100 pmol/mg of SR protein, as compared to 3–4 nmol of phosphorylated intermediate of the Ca^{2+}-pumping ATPase. Thus, its molar ratio of the ATPase was of the order of 0.05, rather than 1 as in the case for phospholamban. In addition (see above), after optimal phosphorylation of the M_r 20 000 proteolipid, a stimulation of the Ca^{2+}-accumulation activity of SR was never observed. Lastly (not shown here), the M_r 20 000 proteolipid was not phosphorylated by cAMP-dependent protein kinases. Taken together, these three observations indicate compellingly that the M_r 20 000 phosphoprotein, in spite of its similarity in physicochemical properties with phospholamban, is different from it. Its function in the Ca^{2+}-transporting cycle of skeletal muscle SR is at the moment a matter of conjecture.

Some Properties of the Membrane-Bound, Calmodulin-Sensitive Protein Kinase Activity. In all likelihood, calmodulin stimulates the kinase that phosphorylates the M_r 57 000, 35 000, and 20 000 polypeptides by interacting with it. In principle then, it should be possible to isolate the kinase on affinity chromatography columns, a procedure that has proven successful for other calmodulin targets (Niggli et al., 1979; Caroni & Carafoli, 1981; Wang et al., 1981). However, the procedure has been unsuccessful in the case of the kinase studied here. The failure was probably due to the hydrophobic character of the enzyme, which can only be solubilized from the membrane with strong detergents. It is very probable that detergents interfere with the binding of calmodulin to the kinase, since even very low concentrations (0.5%) of DOC or of Triton X-100 completely suppressed the calmodulin-dependent phosphorylation of SR proteins (not shown). On the other hand, detergents that did not affect the interaction of calmodulin with the kinase, as inferred from the fact that they did not inhibit the formation of the phosphoproteins, failed to extract the enzyme from the SR membrane. The failure to isolate the kinase on calmodulin affinity chromatography columns cannot thus be considered as conclusive proof that the activator does not interact directly with the kinase. An alternative approach to the problem, and to the more specific question of whether any one of the three calmodulin-sensitive phosphoproteins was the kinase (or its regulatory subunit), was used. Azidocalmodulin was prepared (see Materials and Methods), and it could be estabilished that it activated the kinase (i.e., the formation of the three phosphoproteins) with the same efficiency as normal calmodulin. Then, after photochemical cross-linking was induced between azidocalmodulin and its targets in the SR membrane, the latter was washed free of unbound calmodulin and phosphorylated with [γ-^{32}P]ATP. The denatured phosphorylated SR failed to show on autoradiographed gels additional bands corresponding to phosphoprotein–calmodulin complexes. Thus, it seems clear that the three calmodulin-sensitive SR phosphoproteins do not interact directly with the activator. It is permissible to conclude, then, that none of them is the calmodulin-sensitive kinase or its regulatory subunit.

The nature of the calmodulin-dependent kinase, then, remains obscure. Its kinetic characterization can nevertheless be attempted by using the phosphorylation of its three substrates as a tool. One problem, here, was the presence of the very active Ca^{2+}-ATPase, which consumed most of the added ATP in a short time. Unfortunately, all inhibitors of the ATPase tested also interfered with the kinase (i.e., with the formation of the phosphoproteins) and could thus not be used. By necessity then, experiments of protein phosphorylation in the presence of low concentrations of ATP could only be followed for a very limited time. One additional complication to experiments of this type was contributed by the presence (see below) of an active phosphatase activity in SR membranes.

A first point that could be established was that the phosphorylation of the M_r 57 000 protein is an extremely rapid process: at room temperature, maximal phosphorylation was obtained within 10 s. At 0 °C the half-time for optimal phosphorylation was between 5 and 10 s at optimal Ca^{2+} and calmodulin concentrations. It should be added that the rate of phosphorylation of the 57 000-dalton phosphoprotein is much higher than that recently reported by Campbell & MacLennan (1982). The low rate obtained by these authors can be explained with the different phosphorylation conditions used. Indeed, their characterization was carried out with 10 μM ATP, which is below the K_m of the calmodulin-dependent kinase activity (see below). It is also probably because of the suboptimal conditions used that the phosphorylation level of the 20 000-dalton phosphoprotein was underestimated by

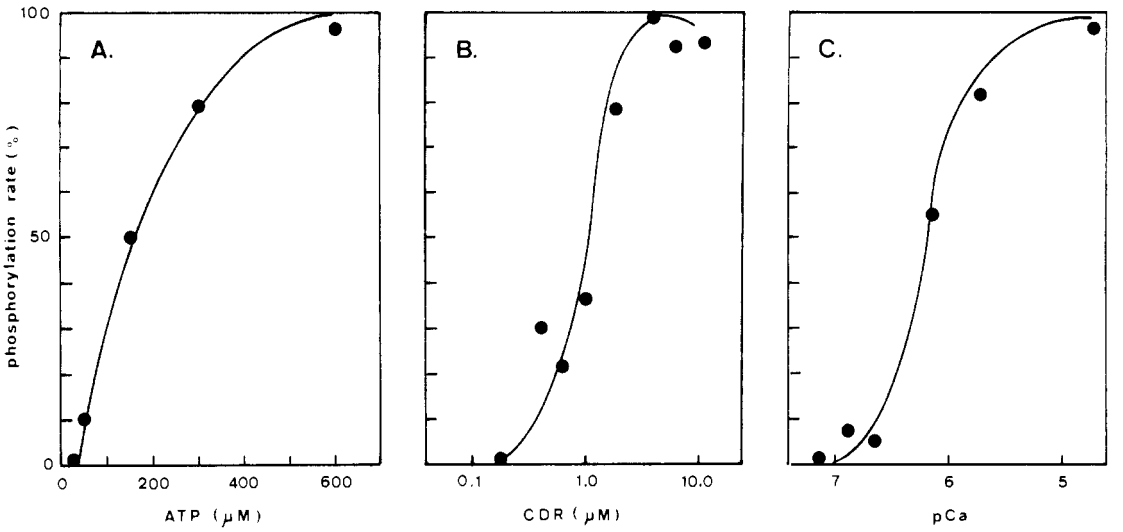

FIGURE 5: (A) ATP dependence of kinase activity. The time dependency of the phosphorylation of SR vesicles in the presence of 2 μM calmodulin, 0.7 μM free Ca, and various amounts of [γ-^{32}P]ATP (with the same specific radioactivity) was studied. The rate of formation of the 20000-dalton phosphoprotein was measured (see Materials and Methods). (B) Calmodulin-dependence of kinase activity. The experiment was carried out as described for panel A with 0.7 μM free Ca, 200 μM [γ-^{32}P]ATP, and various amounts of calmodulin. (C) Ca dependence of kinase activity. The experiment was carried out as described for panel A with 200 μM [γ-^{32}P]ATP, 1.5 μM calmodulin, and various free Ca concentrations.

Campbell & MacLennan (1982), and the failure to observe the calmodulin-dependent phosphorylation of the M_r 35000 protein can be explained in the same way. The velocity of the phosphorylation of the M_r 57000 component made it unsuitable for the study of the kinetics of the kinase. The finding is interesting, however, in that it suggests the possibility that the regulatory process associated with this phosphorylation may, in principle, function on a contraction–relaxation cycle time basis. The phosphorylation of the two other calmodulin-sensitive phosphoproteins was much slower (20–30-s half-time at room temperature) and thus amenable to kinetic analysis. Actually, this half-time might be underestimated, since ATP becomes limiting in 30–60 s, and the phosphorylation of the two proteins may still be less than maximal after this time. With phosphorylation of the M_r 20000 proteolipid as a tool, it could be determined that the K_m(ATP) of the calmodulin-dependent kinase evaluated from the initial rate of appearance of the M_r 20000 phosphoprotein in autoradiographed gels was about 200 μM (Figure 5A). This value seems typical of calmodulin-dependent kinases (Walsh et al., 1979). Figure 5 (panels B and C) also shows that the phosphorylation of the M_r 20000 proteolipid, at optimal ATP concentration, was half-maximally stimulated at 0.7–1.0 μM Ca^{2+} and 1–2 μM calmodulin. The $K_m(Ca^{2+})$ was determined at half-saturating calmodulin concentrations and the K_m-(calmodulin) at half-saturating Ca^{2+} concentrations.

A Calmodulin-Dependent Protein Phosphatase in Skeletal Muscle SR. The three proteins that are phosphorylated by the calmodulin-dependent kinase activity described in the preceeding section will then logically have to be dephosphorylated. Indeed, at the outset of the experiments on the calmodulin-sensitive kinase it was observed that the level of the three phosphoproteins (i.e., the amount of incorporated ^{32}P radioactivity) declined as a function of time once the ATP added to the system became exhausted. This provided a compelling indication for the presence of (a) phosphatase in the preparation.

The first approach used to characterize the hypothetical phosphatase was based on cold ATP chase experiments. The calmodulin-sensitive kinase was allowed to phosphorylate its three substrates in the presence of [γ-^{32}P]ATP (200 μM) for 10–20 s after which time a large excess of unlabeled ATP (1–5 mM) was added. Contrary to expectations, however, the sudden decrease of the specific activity of [γ-^{32}P]ATP in the medium failed to produce, within 2 min, a measurable dephosphorylation of the labeled phosphoprotein. This was taken as an indication that the high concentrations of ATP in the medium inhibited the (hypothetical) phosphatase activity. The inhibitory effect of ATP complicated the study of the phosphatase, since, in the experimental system used, the amount of ATP, after its initial addition to activate the kinase, varied with time, depending essentially on the ATPase activity of the vesicles. The level of ATPase activity, on the other hand, was influenced by some variable of the reaction medium of interest of the characterization of the phosphatase.

The problem was partially overcome by adding hexokinase at the end of the phosphorylation period to rapidly eliminate the ATP remaining in the medium (control experiments established that this occurred within 5 s). Although the procedure was limited by the fact that considerable dephosphorylation of SR took place rapidly after the addition of hexokinase before the medium could be manipulated, it could nevertheless be used to establish some of the properties of the phosphatase. The dephosphorylation of the M_r 57000 phosphoprotein was used as a tool, because it became optimally phosphorylated more rapidly (10–20 s) than the other two substrates of the calmodulin-dependent kinase activity and also because of its better resolution in autoradiographed gels.

A first point that could be established is that the phosphatase requires Ca^{2+} (Figure 6A). The addition of excess EGTA significantly inhibited the dephosphorylation of the M_r 57000 phosphoprotein, whereas the addition of Ca^{2+} in the activity range 0.7–7.5 μM stimulated it. A second point (not shown here) was the lack of effect of F^- (20 mM) or AMP (20 μM) ions, a compelling indication that the phosphatase activity was not due to residues of contaminating phosphorylase phosphatase.

The effect of Ca^{2+} on the dephosphorylation reaction raised the possibility that also the phosphatase in addition to the kinase was calmodulin sensitive. Results suggesting this possibility had been obtained in the course of the study on the kinase, when it was found that the phosphorylation of the M_r 57000 protein became slightly inhibited as the concentration of added calmodulin was increased above 3–5 μM. If a

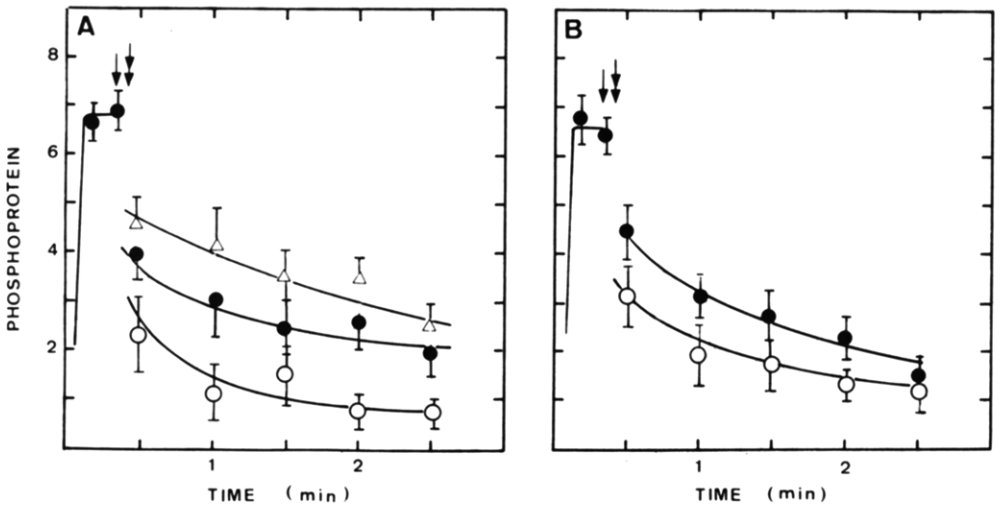

FIGURE 6: Characterization of dephosphorylation kinetics of the 57 000-dalton phosphoprotein. (A) Ca dependency. SR vesicles (1 mg/mL) were phosphorylated in the presence of 1 μM calmodulin, 0.7 mM $CaCl_2$, 1 mM EGTA, and 200 μM [γ-^{32}P]ATP. After 10 and 20 s, aliquots were withdrawn and quenched. At 20 s (↓), hexokinase was added to the incubation mixture (100 units/mL), and at 25 s (double down arrow) the following additions were made: (●) no additions; (△) 5 mM EGTA; (○) 250 μM $CaCl_2$. At sequential time intervals, aliquots were taken, quenched, and characterized by means of polyacrylamide electrophoresis and autoradiography. The level of the 57 000-dalton phosphoprotein was quantitized as described under Materials and Methods and given in the figure with arbitrary units. Results represent mean ● SE for four experiments. (B) Calmodulin dependency. The experiment was carried out as described for panel A. However, at 25 s (double down arrow), the following additions were made: (●) no addition; (○) the calmodulin concentration increased to 4 μM. Results represent mean ± SE for three experiments.

phosphatase activity was activated by the Ca^{2+}–calmodulin complex, however, its affinity for the latter evidently was lower than that of the kinase.

The direct demonstration that calmodulin stimulated a phosphatase was made difficult by the necessity of having calmodulin present in the SR membrane to phosphorylate the (M_r 57 000) substrate of the hypothetical phosphatase. Removal of this calmodulin after completion of the phosphorylation reaction was a lengthy procedure, and on the other hand, the use of the so-called anticalmodulin drugs (e.g., phenothiazines) is open to various criticisms (Roufogalis, 1981). It was nevertheless possible to observe a limited, but significant, stimulation of the dephosphorylation reaction upon addition of extra calmodulin (3 μM) to the medium after removal of ATP by hexokinase (Figure 6B).

More direct evidence for a role of calmodulin in the phosphatase reaction was provided by the experiment illustrated in Figure 7. SR vesicles, phosphorylated for 10 s in the presence of calmodulin, were diluted in a cold medium containing EGTA (to remove most calmodulin from the membrane) and ATP (to inhibit the phosphatase). After centrifugation of SR, the M_r 57 000 protein was still partially phosphorylated, and the conditions affecting its dephosphorylation could now be studied. Ca^{2+} (0.7 μM) did not increase the rate of dephosphorylation, but Ca^{2+} plus calmodulin greatly increased it. This result can be compared with that of Figure 6A, where Ca^{2+} alone increased the rate of dephosphorylation. Since the only difference between the two experiments was the presence of (endogenous) calmodulin in the experiment of Figure 6A, it can be concluded with confidence that SR membranes indeed possess a Ca^{2+}- and calmodulin-sensitive protein phosphatase activity.

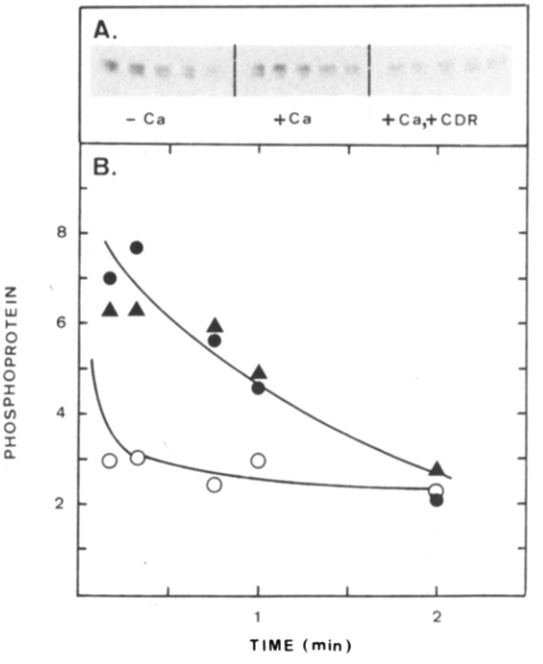

FIGURE 7: Ca–calmodulin dependence of phosphatase activity associated with SR membranes. SR vesicles were phosphorylated with [γ-^{32}P]ATP under optimal conditions by the endogenous calmodulin-dependent kinase. After 10 s, the kinase and phosphatase activities were stopped by dilution in ice-cold medium containing 1 mM ATP and 10 mM EDTA. The vesicles were centrifuged and resuspended (ca. 10 mg/mL) in the basic medium supplemented with 1 mM EGTA. Phosphatase activity was then immediately followed upon 10-fold dilution of the phosphorylated vesicles at room temperature into basic medium containing (●) no Ca, (▲) 0.7 μM free Ca, and (○) 0.7 μM free Ca and 2 μM calmodulin. (A) Autoradiogram illustrating the decay of the 57 000-dalton phosphoprotein. (B) Quantitation of the autoradiogram using arbitrary units.

Discussion

The concentration of calmodulin in muscle has been estimated to be 10–20 μM (Grand et al., 1979). This is a respectable concentration, and it is, therefore, reasonable to expect that the protein may influence the complex biochemical machinery of muscle in ways more than one. Calmodulin is soluble in the cytosol and might, thus, function as in intermediary shuttle in the Ca^{2+} fluxes between the contractile system and sarcoplasmic reticulum. In addition, several enzyme activities of the muscle cell, for example, those involved in the cAMP and glycogen metabolism (LaPorte et al., 1979; Cohen et al., 1978), may be directly regulated by the active Ca–calmodulin complex. On the other hand, calmodulin can

also modulate other reactions of muscle cells by stimulating specific kinases. The stimulation of myosin light chain phosphorylation, which is critical for the initiation of contraction in smooth muscle (Dabrowska et al., 1978), and the stimulation of the phosphorylation of the 22 000-dalton proteolipid phospholamban in cardiac SR (Katz & Remtulla, 1978; LePeuch et al., 1979) are classical examples.

The involvement of calmodulin-dependent kinases in the regulation of the function of skeletal muscle is still an open matter. Calmodulin stimulates myosin light chain phosphorylation also in sarcomeric muscle (Yazawa & Yagi, 1977), but no obvious functional effect is evident. Recent reports that a number of proteins of skeletal muscle SR become phosphorylated in a Ca–calmodulin-dependent way (Chiesi & Carafoli, 1982; Campbell & MacLennan, 1982) have not been directly linked to physiological functions. It has been speculated, however, that the process may be involved in the regulation of Ca^{2+} release from SR membranes.

The role of calmodulin-linked phosphorylations in the regulation of skeletal muscle SR activities has been the subject of the present work. No conclusive demonstration of a specific functional role could be obtained; however, a number of interesting observations have been made on the effect of calmodulin on SR vesicles.

(a) The finding that a calmodulin-dependent kinase phosphorylates three substrates of M_r 20 000, 35 000, and 57 000, which are an integral part of SR membranes, has been confirmed and extended. Interestingly, the SR-phosphorylated proteins are among the major substrates of calmodulin-dependent kinases in skeletal muscle cells (Figure 1).

(b) The M_r 20 000 phosphoprotein has been characterized as an acidic proteolipid. This finding is of particular interest, since several other proteolipids of key importance for the regulation of ion fluxes through biological membranes have been discovered. The function of these proteolipids, e.g., the 22 000-dalton phospholamban of cardiac SR (LePeuch et al., 1979) or the 23 000-dalton calciductin of cardiac sarcolemma (Rinaldi et al., 1981), depends on their phosphorylation state. In spite of the close similarities, the 20 000-dalton phosphoprotein of skeletal muscle SR is not identical with cardiac phospholamban nor with calciductin. Its physiological role is at the moment unknown.

(c) It has been demonstrated also that the 57 000-dalton substrate of the calmodulin-dependent kinase is a relatively hydrophobic polypeptide, since it cannot be extracted from SR membranes by low concentrations of the detergent Lubrol. The use of a special electrophoretic procedure at neutral pH and the study of the extraction characteristics in lubrol have permitted establishment that the 57 000-dalton phosphoprotein is distinct from both calsequestrin and the glycoprotein, which have similar molecular weights. This phosphoprotein is probably identical with the 60 000-dalton SR protein, which becomes phosphorylated in a calmodulin-dependent way (Campbell & MacLennan, 1982). That the 60 000-dalton phosphoprotein is not calsequestrin was shown by the lack of reactivity with calsequestrin-directed antibodies. That it is not the 53 000-dalton glycoprotein was demonstrated by the fact that it is not digested by endo-β-*N*-acetylglucosaminidases. The difference in M_r between 57 000 given here and 60 000 given by others (Campbell & MacLennan, 1982) is likely to be linked to experimental differences in the calibration procedure of the gels.

(d) A calmodulin-dependent kinase that is strongly associated with the SR membranes has been demonstrated. The kinase has not been identified, but the data have ruled out the possibility that one of the phosphoproteins described might represent the kinase or, at least, its regulatory subunit. This is so because none of the three phosphoproteins interact directly with azidocalmodulin.

The findings made, however, do not rule out the possibility that one (or more) of the three phosphoproteins is the catalytic subunit of the calmodulin-dependent kinase, which does not interact with calmodulin. In this case, the most probable candidate would be the M_r 57 000 protein, which is phosphorylated very rapidly ($t_{1/2}$ = 5–10 s at 0 °C), as is typical of many autophosphorylating kinases. If, on the other hand, the 57 000-dalton phosphoprotein were not the catalytic subunit of the kinase, it would be ideally qualified for a role in a regulatory process known to occur within the (millisecond) time range of the contraction–relaxation cycle.

The kinetic study of the calmodulin-dependent kinase activity has shown a relatively low affinity of the kinase enzyme for ATP (K_m = 200 μM). It has also indicated that the kinase, at the assumed micromolar concentration of calmodulin in muscle cytosol (Grand et al., 1979), can be turned on or off by Ca^{2+} fluctuations in the physiological range [K_m(Ca) = 0.7 μM at 1.5 μM calmodulin concentration].

(e) Most interestingly, a calmodulin-dependent phosphatase activity has been discovered in SR membranes. The activity is not extractable by EGTA, showing that it is not a soluble phosphatase like calcineurin, the first calmodulin-dependent phosphatase discovered (Stewart et al., 1982).

It has emerged from the results presented that the level of phosphorylation of protein substrates like the M_r 57 000 protein of SR membranes [and thus, presumably the level of activity of the unknown function(s) modulated by it] is under the control of kinases and phosphatases, which are both calmodulin sensitive. Since these opposing activities are sensitive to the Ca–calmodulin complex, rather than to calmodulin alone, and since the level of calmodulin in the cytosol is not likely to change rapidly, it is logical to presume that the respective level of activity of the kinase and the phosphatase is controlled by the cytosolic Ca^{2+} level. The central problem left open by the results presented in this study is, of course, the physiological significance of the calmodulin-directed phosphorylation/dephosphorylation cycle of the three SR protein substrates. The hypothesis that the process may somehow be involved in the release of Ca^{2+} from SR (Chiesi & Carafoli, 1982; Campbell & MacLennan, 1982) is attractive and is now being tested in our laboratory.

Registry No. ATP, 56-65-5; calcium, 7440-70-2; protein kinase, 9026-43-1; phosphoprotein phosphatase, 9025-75-6.

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Partial Amino Acid Sequence of a Rabbit Immunoglobulin Light Chain of Allotype b5†

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ABSTRACT: The amino acid sequence of a rabbit immunoglobulin light chain of allotype b5 has been nearly completed. A comparison of its structure with that of light chains of allotypes b4, b6, and b9 confirms that the constant regions of these various κ chains differ by 20–35%. The substitutions are clustered in parts of the second half of the chain, and the b5 form bears more resemblance to the b6 chain than to any other, in good agreement with previous serological data. The analysis of the variable region reveals the existence of certain allotype-associated residues which have also been reported in other b5 chains, but not in proteins of the other allotypes. An examination of the rabbit light chain sequences between positions 96 and 107 suggests that this portion of the chain may be encoded separately by a joining "J" DNA segment, as has been described previously for murine and human immunoglobulins. In the rabbit, however, these J_κ regions appear to differ from one allotype to another. Together with the extensive variations of the constant regions, these data suggest that the rabbit κ gene organization more closely resembles the murine λ system (four different C_λ genes each flanked by its J segment) than the murine κ system (a single C_κ gene).

Serological studies have documented the existence of four major domestic rabbit κ light chain allotypes: b4, b5, b6, (Oudin, 1960), and b9 (Dubiski & Muller, 1967). Breeding studies suggest that these allotypes are the products of codominant allelic structural genes.

Amino acid sequence studies have revealed amino acid substitutions in the light chain variable region (V_L)[1] which seem to correlate with the allotypes: statistical differences in the expression of particular N-terminal sequences in light chains of different allotypes (Waterfield et al., 1973) have been confirmed by the analysis of individual chains, mostly of allotype b9 (Fraser et al., 1978).

Sequence studies of the light chain constant region (C_L) indicate between 20 and 35% differences between chains of allotypes b4, b6, and b9 (Zeeuws & Strosberg, 1975; Farnsworth et al., 1976; Emorine et al., 1979). A small number of substitutions appear to distinguish constant regions of the same allotype (Sogn & Kindt, 1976; Strosberg et al., 1974; Emorine et al., 1979). Data for the b5 allotype have been

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[1] Abbreviations: V_L, variable region of light chain; C_L, constant region of light chain; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; IAA, iodoacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PITC, phenyl isothiocyanate; TFA, trifluoroacetic acid; DABITC, (dimethylamino)azobenzene isothiocyanate; Gdn·HCl, guanidine hydrochloride; TPCK, tosylphenylalanyl chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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