

Isolation of the calmodulin-dependent protein kinase system from rabbit skeletal muscle sarcoplasmic reticulum

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A calmodulin-dependent protein kinase system from the sarcoplasmic reticulum was dissolved in Nonidet P40, adsorbed to a CaM affinity column in the presence of Ca²⁺ and eluted in the presence of EGTA. The purified fraction contained major proteins of 60 and 20 kDa and minor components of 89 and 34 kDa, all of which were phosphorylated with dependencies on Ca²⁺, CaM, ATP and pH similar to those observed in the sarcoplasmic reticulum. Differences in the phosphopeptides produced by partial proteolysis of the individual phosphoproteins indicated that they are distinct entities. ¹²⁵I-CaM labeled only the 60 kDa protein, suggesting that it is a kinase.

Protein phosphorylation; Sarcoplasmic reticulum; Calmodulin

1. INTRODUCTION

A calmodulin-dependent protein kinase system has been identified in skeletal muscle sarcoplasmic reticulum which leads to phosphorylation of membrane proteins of 85, 60, 34 and 20 kDa [1–3]. The phosphoproteins are minor components of the sarcoplasmic reticulum, the 60 kDa component being present at a concentration 1/50 that of the Ca²⁺-ATPase [1] and the others at lower concentrations. The function of the phosphorylation system is still unknown, although the question of whether it could be involved in Ca²⁺ release has been explored [1–5]. Here, we report the isolation of the calmodulin-dependent protein kinase system in an active form, and show that the 60 kDa protein is the calmodulin-binding subunit and may, therefore, be the kinase that undergoes autophosphorylation.

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2. MATERIALS AND METHODS

2.1. Purification of the kinase system

Sarcoplasmic reticulum membranes were isolated and extracted with EGTA as in [1,2]. The EGTA-extracted membranes were suspended at 10 mg/ml in a solution of 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, and 1 mM histidine. The detergent NP40 was added to 0.7% from a 10% stock solution and the mixture incubated on ice for 15 min. After centrifugation for 45 min at 120000 × g, more than 80% of the calmodulin-dependent protein kinase activity was found in the supernatant. Calmodulin was purified from bovine cerebral cortex by the method of Teo et al. [6] and coupled to Sepharose CL-4B using divinyl sulphone [7]. The solubilized sarcoplasmic reticulum proteins were loaded onto the calmodulin-Sepharose CL-4B column equilibrated with a solution of 0.15 M NaCl, 0.1% NP40 and 50 mM Tris-HCl, pH 7.4 (buffer A), containing 0.1 mM CaCl₂ and the column washed extensively with buffer A. The calmodulin-binding proteins were then eluted with buffer A, without CaCl₂, but containing 2 mM EGTA.

2.2. Peptide mapping

Proteins were phosphorylated at pH 6.0 as in [1,2] and localized in gels by autoradiography. Gel slices containing the 89, 60, 34 and 20 kDa phosphoproteins were proteolyzed partially with 5 µg *S. aureus* V8 protease as described by Cleveland et al. [8] and visualized by autoradiography.

2.3. Identification of calmodulin binding proteins

Calmodulin-binding proteins were identified in the sar-

coplasmic reticulum and the purified kinase system by a 125 I-calmodulin overlay technique [9,10].

3. RESULTS

3.1. Purification of the calmodulin-dependent protein kinase system of sarcoplasmic reticulum

Fig.1 (lane 1) illustrates solubilization of the calmodulin-dependent protein kinase system, consisting of phosphoproteins of 89, 60, 34 and 20 kDa, upon addition of 0.7% NP40 to EGTA-extracted sarcoplasmic reticulum vesicles. The kinase system was subjected to affinity chromatography on a calmodulin column. Most of the protein appeared in the void volume but about 80% of the protein which could be phosphorylated was bound to the column (fig.1, lane 2). Proteins of the kinase system that appeared in the void volume were bound during a second passage

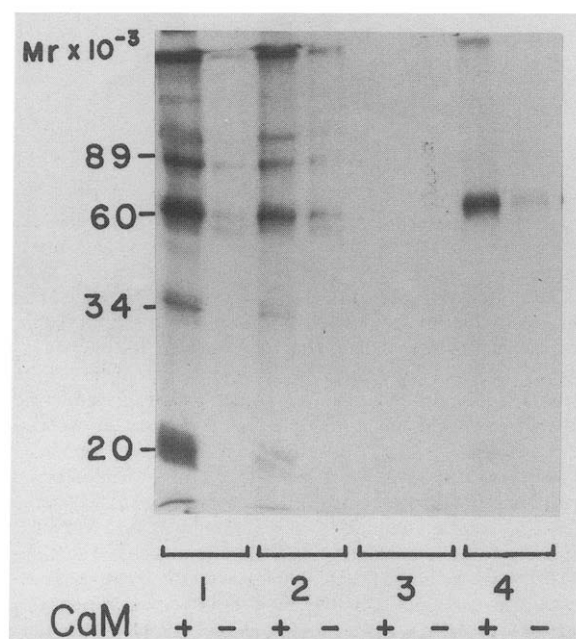


Fig.1. Purification of the protein kinase system of the sarcoplasmic reticulum on a calmodulin affinity column. Protein fractions were separated on SDS-polyacrylamide gels and subjected to autoradiography and phosphorylated in the presence and absence of calmodulin. An autoradiogram of the phosphoprotein composition is presented. 1, proteins solubilized in NP 40 prior to application to the calmodulin affinity column; 2, material passing through the calmodulin affinity column; 3, wash fractions; 4, fraction eluted from the column with EGTA.

Table 1

Substrate specificity of Ca^{2+} /calmodulin-dependent protein kinase

Substrate	Phosphorylation (pmol/mg)
60 kDa protein	1052
89 kDa protein	134
20 kDa protein	62
Casein	24.4
Protamine	0
Histone	0

The purified calmodulin-dependent protein kinase was phosphorylated in the presence of Ca^{2+} and calmodulin with ^{32}P ATP. The phosphoproteins were separated in SDS gels and ^{32}P incorporation into each protein band was determined as described in section 2. Each substrate (100 μg protein) was phosphorylated with 0.5 μg purified kinase preparation in the presence of Ca^{2+} and calmodulin

through the calmodulin column (not shown). Proteins eluted in the wash fraction could not be phosphorylated (fig.1, lane 3), but calmodulin-dependent phosphorylation of the 60 and 20 kDa proteins was seen in a small protein peak eluted with EGTA (fig.1, lane 4). We have observed specific activities of ^{32}P incorporation into the 60 kDa protein in this fraction as high as 1 nmol/mg protein (table 1).

3.2. Polypeptide composition and autophosphorylation of the calmodulin-dependent protein kinase system

The protein composition of the fraction eluted with EGTA from the calmodulin affinity column is shown in fig.2 (lane A). A major protein doublet of 60 kDa and a major band at 20 kDa were seen in the silver-stained SDS gel while minor bands were observed in different positions. The 60 kDa protein doublet may be due to differential migration in SDS gels of phosphorylated and unphosphorylated forms of the polypeptide. The 60 kDa protein was the major phosphorylated species, but calmodulin-dependent phosphorylation of proteins of 89, 34 and 20 kDa was also seen, at much lower levels (fig.2, lane B; fig.3; table 1).

3.3. Phosphopeptide analysis

To determine whether there was a structural relationship between the various phosphoproteins, their structures were compared by analysis of par-

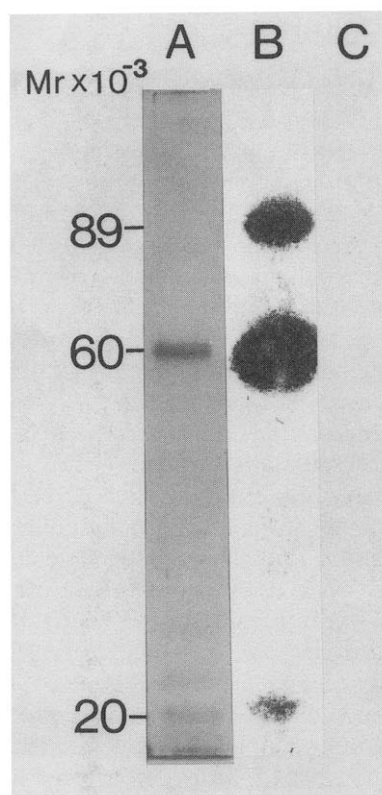


Fig.2. Polypeptide composition and autophosphorylation of calmodulin-affinity purified proteins. Proteins eluted with EGTA from the calmodulin affinity column were phosphorylated with [32 P]ATP in the presence and absence of calmodulin, and separated by SDS-polyacrylamide gel electrophoresis. A, silver staining of the polypeptides purified on the calmodulin column; B, autoradiogram of the purified calmodulin-binding proteins phosphorylated in the presence of Ca^{2+} and calmodulin; C, phosphorylation of the calmodulin-binding proteins in the absence of calmodulin.

tial proteolytic products using *S. aureus* V8 protease [8]. The phosphopeptide patterns generated in this way are shown in fig.3. Differences could be seen in the phosphopeptide patterns of the 89, 60, 34 and 20 kDa proteins, demonstrating that the proteins were unique and not derived from each other by proteolysis.

3.4. ^{125}I -calmodulin binding to the calmodulin-dependent protein kinase system

We attempted to identify the polypeptide that confers the Ca^{2+} /calmodulin dependence on the enzyme system by measuring the binding of ^{125}I -calmodulin to sarcoplasmic reticulum proteins,

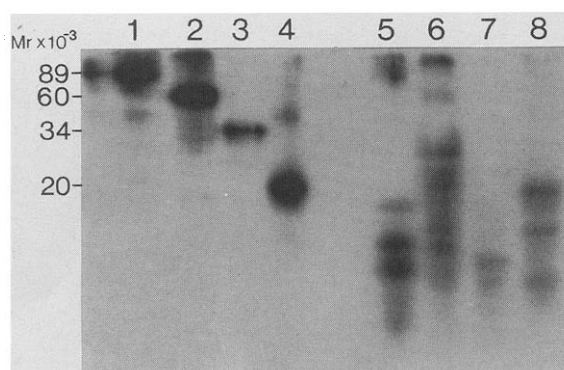


Fig.3. Partial proteolysis of autophosphorylated calmodulin-dependent phosphoproteins. Purified proteins ($1.0\ \mu\text{g}$) were autophosphorylated with [32 P]ATP in the presence of calmodulin, separated on SDS gels, stained, dried and subjected to autoradiography to localize the polypeptides. Gel pieces containing ^{32}P -labeled phosphoproteins were subjected to limited proteolysis as described in the text, using *S. aureus* V8 protease. Lanes 1–4, undigested phosphoproteins of the kinase system; 5, 89 kDa phosphoprotein digest; 6, 60 kDa phosphoprotein digest; 7, 34 kDa phosphoprotein digest; 8, 20 kDa phosphoprotein digest. Major differences between phosphoproteins are indicated by arrowheads.

separated in denaturing gels and renatured as described in [9]. The results are shown in fig.4. In the presence of EGTA, ^{125}I -calmodulin did not bind to any of the proteins in the sarcoplasmic reticulum, the EGTA extract or the purified fraction. In the presence of Ca^{2+} , ^{125}I -calmodulin was bound to a protein band of 60 kDa in the sarcoplasmic reticulum membrane and in the purified fraction. In addition, a protein of 140 kDa in the purified fraction was found to be weakly labelled under these conditions. Our attempts to measure autophosphorylation of these separated protein bands after renaturation in the presence of Ca^{2+} , CaM and [^{32}P]ATP were unsuccessful.

3.5. Characterization of calmodulin-dependent protein phosphorylation

Calmodulin-dependent phosphorylation of the different proteins had the same characteristics in the isolated fraction as in the sarcoplasmic reticulum. Phosphorylation of the 60 kDa protein preceded phosphorylation of 89, 34 or 20 kDa proteins. In the purified preparation we did not observe any dephosphorylation of the phosphoproteins, indicating that the preparation was free of phosphatase activity. When

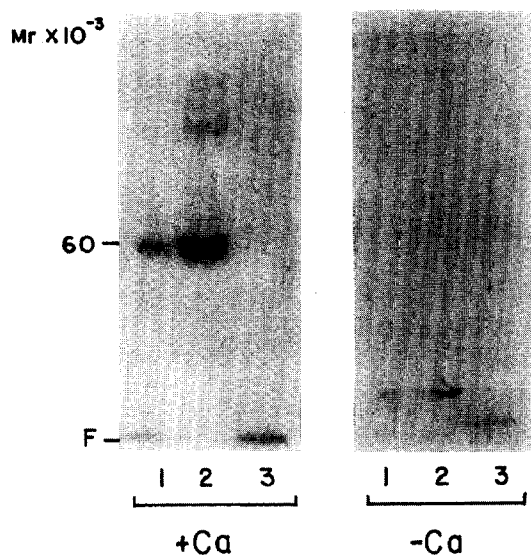


Fig.4. Identification of ^{125}I -calmodulin-binding proteins. Sarcoplasmic reticulum membranes (lane 1), purified kinase system (lane 2) and EGTA supernatant (lane 3), were separated by SDS-polyacrylamide gel electrophoresis, and ^{125}I -calmodulin binding was analysed as described in section 2. An autoradiogram of ^{125}I -calmodulin binding in the presence of Ca^{2+} (+ Ca^{2+}) or presence of EGTA (- Ca^{2+}) is shown.

phosphorylation was carried out at various concentrations of $[\text{}^{32}\text{P}]\text{ATP}$, maximal phosphorylation occurred at $50\text{ }\mu\text{M}$ ATP and further increases in ATP concentration resulted in significant inhibition of ^{32}P incorporation. The purified protein kinase system had an absolute requirement for calmodulin and Ca^{2+} for activation, with maximal phosphorylation occurring at about $0.6\text{ }\mu\text{M}$ calmodulin and the half-maximal level being reached at about $0.1\text{ }\mu\text{M}$. In the presence of $0.6\text{ }\mu\text{M}$ calmodulin, phosphorylation of all three proteins increased dramatically when the free Ca^{2+} concentration was raised from 0 to $0.25\text{ }\mu\text{M}$ but further elevations in free Ca^{2+} led to inhibition of protein phosphorylation. About 25% inhibition of phosphorylation was observed at $1\text{ }\mu\text{M}$ Ca^{2+} vs that seen for $0.25\text{ }\mu\text{M}$ Ca^{2+} . Calmodulin-dependent ^{32}P incorporation into the purified 89, 60, 34 and 20 kDa proteins was maximal at pH 6.0, and decreased to 1/4 as the pH was lowered to 5.5 or 1/2 as the pH was raised to 8. Casein served as a substrate for the purified kinase while pro-tamine and histones did not (table 1).

4. DISCUSSION

Analysis of the purified calmodulin-dependent protein kinase system from the skeletal muscle sarcoplasmic reticulum membrane indicates that it consists of at least four polypeptides of 89, 60, 34 and 20 kDa. Only the 60 kDa protein was able to bind ^{125}I -calmodulin suggesting that it contains a calmodulin-binding domain and is the calmodulin-dependent kinase which phosphorylates both itself and other components of the system. Since the other proteins were unable to bind calmodulin, we can only account for their copurification on the calmodulin column if they existed as a complex with the 60 kDa protein. The apparent stoichiometry between the 60 and 20 kDa proteins on the basis of protein staining was about 2:1 but we were unable to determine the stoichiometry of the 89 and 34 kDa proteins because of their low quantities in the preparation.

In considering the physiological role of the calmodulin-dependent protein kinase system, it should be noted that the system is an intrinsic component of the sarcoplasmic reticulum, since it can only be extracted with detergents. Since the Ca^{2+} -uptake mechanism in sarcoplasmic reticulum is not affected by calmodulin, we have previously proposed that the calmodulin-dependent phosphorylation of the 60 kDa protein may be important in the regulation of Ca^{2+} release from sarcoplasmic reticulum [4]. Kim and Ikemoto [5] have shown a correlation between Ca^{2+} release and the phosphorylation of the 60 kDa protein and Meissner [11] has found that calmodulin inhibits the Ca^{2+} -release channel. The availability of the purified kinase system should allow experimental evaluation of any role for this system in the regulation of Ca^{2+} release.

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