A 60 kDa polypeptide of skeletal-muscle sarcoplasmic reticulum is a calmodulin-dependent protein kinase that associates with and phosphorylates several membrane proteins

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Activation of a calmodulin (CaM)-dependent protein kinase associated with rabbit skeletal-muscle sarcoplasmic reticulum (SR) results in the phosphorylation of polypeptides of 450, 360, 165, 105, 89, 60, 34 and 20 kDa. Radioligand-binding studies indicated that a membrane-bound 60 kDa polypeptide contained both CaM- and ATP-binding domains. Under renaturing conditions on nitrocellulose blots, the 60 kDa polypeptide of the membrane exhibited CaM-dependent autophosphorylation activity, suggesting that it was the CaM-dependent protein kinase of SR. Ca^{2+}/CaM-independent autophosphorylation of polypeptides of 62 and 45 kDa was found to occur in the light SR, whereas the Ca^{2+}/CaM-dependent autophosphorylation activity was enriched in the heavy SR. Both these kinase activities were absent from transverse tubules, although these membranes were enriched in CaM-binding polypeptides of 160, 100 and 80 kDa. In the absence of Ca^{2+}, CaM bound to a 33 kDa polypeptide of the membrane. The purified ryanodine receptor was not phosphorylated by the purified CaM kinase, although it was a substrate for protein kinase C. Affinity-purified antibodies to brain CaM kinase II cross-reacted with the 60 kDa polypeptide in Western blots and immunoprecipitated the 60 kDa polypeptide, along with the 360, 105, 89, 34 and 20 kDa phosphoproteins, from Nonidet-P-40-solubilized SR membranes. Antibodies raised against the 60 kDa kinase polypeptide did not cross-react with the other phosphoproteins, suggesting that these polypeptides were distinct and unrelated. Subcellular distribution of the 60 kDa kinase indicated the specific association of the polypeptide with the junctional-face membrane of SR. The CaM-dependent incorporation of ^{32}P into various membrane proteins was inhibited by the CaM kinase II fragment (290–309), with an IC_{50} value of 2 nM for the inhibition of incorporation into the 60 kDa kinase polypeptide. Recent studies [Wang and Best (1992) Nature (London) **359**, 739–741] have shown that a CaM kinase activity intrinsic to the membrane can inactivate the Ca^{2+}-release channel of skeletal muscle SR. Since our results demonstrate that the 60 kDa polypeptide of SR is a CaM-dependent protein kinase, we suggest that this kinase, through its associations, may be responsible for gating the Ca^{2+}-release channel.

INTRODUCTION

In skeletal muscle, depolarization of the sarcolemmal membrane is thought to be coupled to the release of intracellular Ca^{2+} from the sarcoplasmic reticulum (SR), a complex network of membranes that surrounds each myofibril and controls the level of intracellular free Ca^{2+} (Fleischer and Inui, 1989; Rios and Pizarro, 1991). The electrical signal transmission seems to occur across the triad junction, where a large number of dihydropyridine (DHP) receptors and foot structures have been localized (Franzini-Armstrong and Nunzi, 1983; Block et al., 1988; Catterall, 1991). A depolarization-induced conformational change in the DHP receptor is thought to be transmitted to the foot-structure ryanodine receptor to initiate Ca^{2+} release. These foot structures have been identified as the Ca^{2+}-release channels of the SR, which have been cloned and extensively characterized at the biochemical and electrophysiological levels (Lytton and MacLennan, 1991; Williams, 1992). The Ca^{2+}-release channel of skeletal-muscle SR can be induced to open or close by Ca^{2+} (Endo et al., 1968; Fabiato and Fabiato, 1975; Baylor et al., 1983; Meissner, 1986; Hymel et al., 1988; Lai et al., 1988). The exact mechanisms by which the Ca^{2+}-release channel is gated by Ca^{2+} remains unclear.

It has been postulated that changes in the level of phosphorylation of SR proteins might regulate changes in ionic permeability of the SR membranes (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; MacLennan et al., 1984; Tuana and MacLennan, 1984; Kim and Ikemoto, 1986; Morii et al., 1987). More specifically, a calmodulin (CaM)-dependent protein kinase activity that has been shown to associate with rabbit skeletal-muscle SR was proposed to gate the Ca^{2+}-release channel in response to increased Ca^{2+} activity during muscle activation (MacLennan et al., 1984). The CaM kinase phosphorylates several proteins and is a minor component of the SR, with a stoichiometry resembling the ryanodine receptor rather than the Ca^{2+}-ATPase (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Tuana and MacLennan, 1984). The isolated CaM kinase complex consists of the 89, 60, 34 and 20 kDa phosphoproteins, and the 60 kDa polypeptide appears to be the major CaM-binding polypeptide (Tuana and MacLennan, 1988). The CaM-dependent phosphorylation of a 60 kDa polypeptide has been correlated with the extent of inhibition of Ca^{2+} release from SR (Kim and Ikemoto, 1986). Recent studies using the patch-clamp technique have implied that a protein kinase activity intrinsic to the SR membrane could close the Ca^{2+}-release channel (Wang and Best, 1992). The closure of the Ca^{2+}-release channel

Abbreviations used: SR, sarcoplasmic reticulum; DTT, dithiothreitol; CaM, calmodulin; NP40, Nonidet P-40; DHP, dihydropyridine; t-tubule, transverse-tubule.

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by phosphorylation could be prevented by a CaM kinase II inhibitory peptide, suggesting the CaM kinase activity of SR was involved in channel gating. In addition, a Ca^{2+}/CaM-independent kinase activity associated with SR has also been described to phosphorylate polypeptides of 62, 42 and 20 kDa. Since the identity and the mechanism of action of the CaM kinase and the Ca^{2+}/CaM-independent kinase of skeletal muscle SR remain unclear, we have undertaken to characterize these protein kinases further.

EXPERIMENTAL

Materials

[γ-^{32}P]ATP (3000 Ci/mmol) and Bolton–Hunter-^{125}I-labelled CaM were purchased from DuPont–New England Nuclear. Nitrocellulose membranes (0.2 μm pore size) were obtained from Technical Marketing (Ottawa, Ontario, Canada). The CaM–agarose affinity column was purchased from Sigma Chemical Co. All electrophoresis chemicals, molecular-mass standards and alkaline-phosphatase-conjugated anti-rabbit IgG were purchased from Bio-Rad. ATP was obtained from Boehringer Mannheim. X-ray film for autoradiography was purchased from Eastman-Kodak. All other chemicals were of reagent grade and obtained from Sigma or other local suppliers.

Preparation of rabbit skeletal-muscle fractions

Microsomal membranes were prepared from fast-twitch skeletal muscle from the backs of New Zealand White rabbits as described by Meissner (1984). Light and junctional SR fractions were prepared as described by Chu et al. (1988) and junctional-face membranes were isolated (Costello et al., 1988). For further comparison, a transverse-tubule-enriched fraction was prepared as described by Doucet and Tuana (1991). Proteolytic inhibitors were added to all buffers, as follows: 1 mM benzamidine, 1 mM iodoacetamide, 0.5 μM pepstatin A and 0.3 mM phenylmethanesulphonyl fluoride.

Isolation of the CaM kinase complex

Skeletal-muscle SR membranes were isolated and extracted with EGTA as described by Tuana and MacLennan (1984). The kinase complex was isolated as described by Tuana and MacLennan (1988), with the following modifications. The EGTA-extracted membranes were suspended in 10 mM Tris/HCl buffer, pH 7.4, and solubilized in 0.2% Nonidet P-40 (NP40) for 15 min on ice. After centrifugation at 120000 *g* for 45 min at 4 °C, the supernatant was adjusted to 1 mM free Ca^{2+} and loaded on a CaM–agarose affinity column equilibrated with 0.15 M NaCl, 0.1% NP40 and 50 mM Tris/HCl, pH 7.4 (buffer A), containing 0.1 mM $CaCl_2$. After incubation for 60 min at 4 °C, the column was washed extensively with buffer A and the CaM-binding proteins were eluted with buffer A containing 2 mM EGTA. The CaM kinase from cardiac SR was isolated in a similar manner (B. S. Tuana, unpublished work).

Phosphorylation assay

Phosphorylation of samples was carried out at room temperature in the kinase buffer described by Campbell and MacLennan (1982), i.e. 50 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 10 mM NaF, 0.2 mM EGTA, 0.5 mM $CaCl_2$, 10–50 μM [γ-^{32}P]ATP, in the absence or presence of 0.6 μM CaM. After incubation for 90 s, the reactions were terminated with SDS loading buffer, containing 8 M urea, 3% SDS, 100 mM dithiothreitol (DTT), 0.005% Bromophenol Blue and 70 mM Tris/HCl, pH 6.7. Samples were boiled at 100 °C for 2 min and analysed quantitatively by SDS/PAGE. Phosphorylated polypeptides were localized by autoradiography, cut out of the dried gel, and the radioactivity was quantified by liquid-scintillation counting.

Identification of CaM-binding proteins

CaM-binding proteins were identified by an overlay technique essentially as described by Carlin et al. (1981). Samples were separated by SDS/PAGE and transferred to nitrocellulose. The nitrocellulose was blocked for 60 min with powdered milk (Carnation) in 200 mM NaCl and 20 mM Tris/HCl, pH 7.2, and incubated overnight with ^{125}I-CaM in the presence of 1 mM $CaCl_2$ or 2 mM EGTA. The dried membranes were exposed to X-ray film for autoradiography.

CaM-dependent autophosphorylation

Proteins were separated by SDS/PAGE in 10% polyacrylamide and electroblotted to nitrocellulose (Doucet and Tuana, 1991), except that methanol was omitted from the transfer buffer. The autophosphorylation assay was carried out essentially as described by Celenza and Carlson (1986) with the following modifications. The nitrocellulose was blocked with powdered milk in 30 mM Hepes, pH 7.5. Proteins bound to the nitrocellulose were denatured for 1 h at 25 °C with 7 M guanidine/HCl/50 mM DTT/2 mM EDTA/0.25% powdered milk in 50 mM Tris/HCl (pH 8.3) and were then allowed to renature for 16 h at 4 °C in 50 mM Tris/HCl (pH 7.5) containing 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP40 and powdered milk. The nitrocellulose was treated with 5% powdered milk in 30 mM Hepes, pH 7.5, for 30 min at 25 °C and then incubated with the kinase buffer, [γ-^{32}P]ATP and 0.25% powdered milk in the absence or presence of 0.6 μM CaM. After several washings with 30 mM Hepes, pH 7.5, and 0.25% powdered milk at 25 °C, the nitrocellulose was washed for 10 min at 4 °C with 1 M KOH solution to remove non-specifically bound [γ-^{32}P]ATP and dried for autoradiography.

ATP photoaffinity labelling

Photoaffinity labelling of the isolated kinase complex was carried out by the method of Nairn and Greengard (1987). Assay conditions were similar to those used for the phosphorylation, except that 10 nM azido-ATP replaced [γ-^{32}P]ATP. Samples were incubated at room temperature for 5 min in the absence or presence of excess unlabelled ATP, then placed on ice and irradiated for 2 min. The reactions were terminated with SDS loading buffer. The samples were subjected to SDS/PAGE and autoradiography.

Preparation of antibodies against the 60 kDa polypeptide

The 60 kDa CaM-binding protein was isolated from a CaM affinity column and gel-purified. A 10 μg portion of purified 60 kDa polypeptide was injected into guinea pigs to raise antibodies, and a 5 μg boost was administered at 2–4-week intervals. Serum from immunized guinea pigs and preimmune serum from guinea pigs was obtained and stored at −70 °C until use.

Immunoprecipitation assay

Skeletal-muscle microsomes were phosphorylated as described above in the absence or presence of 0.6 μM CaM. The solubilized fractions were incubated for 60 min at 25 °C or overnight at 4 °C with the affinity-purified polyclonal antibodies to the brain multifunctional CaM kinase II. The antigen–antibody complexes were isolated by adsorption to Protein A–agarose. The antigen–antibody–Protein A–agarose pellets were washed three times in 200 mM NaCl/0.5% NP40/10 mM Tris/HCl, pH 7.4, and then once in the same buffer without detergent. NP40-solubilized fractions were also subjected to a pre-adsorption step using control rabbit serum and Protein A–agarose. The proteins were eluted from the Protein A–agarose beads by boiling for 5 min with SDS loading buffer and subjected to SDS/PAGE and autoradiography.

SDS/PAGE, electroblotting and immunostaining

Protein samples were subjected to electrophoresis in a highly porous SDS/PAGE (10%-acrylamide) system (Doucet et al., 1990), which permits rapid and efficient transfer of proteins to nitrocellulose while maintaining a broad degree of resolution. The proteins resolved on gels were transferred to nitrocellulose sheets electrophoretically (Towbin et al., 1979). The anti-(brain CaM kinase II) antibody was used at a dilution of 1:5000 (60 min incubation), and the reaction was made visible with alkaline-phosphatase-conjugated anti-rabbit IgG. When silver staining was desired, the method of Morrissey (1981) was used. Autoradiography of dried gels was performed with Kodak X-Omat AR film and a Dupont Cronex Lightning Plus enhancing screen.

Protein assay

Protein concentrations were determined by the method of Markwell et al. (1978).

RESULTS

CaM-binding proteins in subcellular fractions from skeletal muscle

Various subcellular fractions of rabbit skeletal muscle were separated by SDS/PAGE and transferred to nitrocellulose, which was incubated with ^{125}I-CaM to identify CaM-binding polypeptides. The resulting autoradiogram is shown in Figure 1. In the presence of calcium (Figure 1a), a 60 kDa polypeptide, enriched in the heavy-SR membrane preparations (lane 4), was the predominant CaM-binding protein. An 80 kDa CaM-binding polypeptide which was present in lower amounts was also detected in the heavy-SR preparations. CaM-binding polypeptides of 100 and 160 kDa also specifically bound CaM in the presence of Ca^{2+} and were enriched in the transverse-tubule fractions (lane 2). In the presence of EGTA (Figure 1b), a 33 kDa polypeptide present in the light membrane fraction (lane 1) was labelled by ^{125}I-CaM.

Identification of the polypeptides exhibiting autophosphorylation activity in SR membranes

The CaM-dependent phosphorylation activity associated with the sarcoplasmic reticulum of skeletal muscle, as evidenced by autoradiography, is shown in Figure 2(a). SR membranes were phosphorylated with [γ-^{32}P]ATP in the presence (lane 1) or

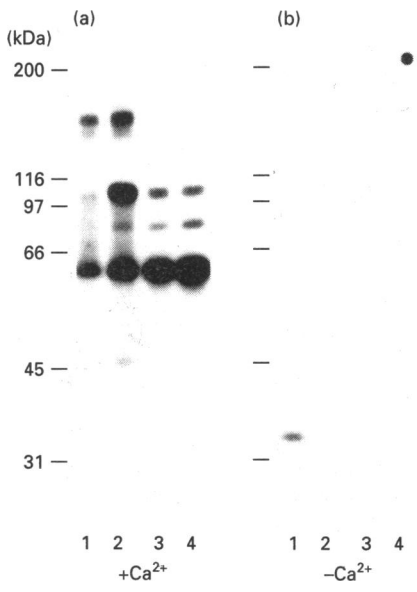

Figure 1 Identification of ^{125}I-CaM-binding proteins in rabbit skeletal muscle

Samples of various skeletal-muscle fractions were separated by SDS/PAGE and transferred to nitrocellulose. The nitrocellulose was incubated for 16 h at room temperature with ^{125}I-CaM in the presence of (**a**) 1 mM Ca^{2+} or (**b**) 2 mM EGTA. The dried nitrocellulose membranes were exposed for autoradiography: light membrane fraction (lane 1); transverse tubules (lane 2); light SR (lane 3); heavy SR (lane 4). Numbers at the left-hand side of many Figures indicate molecular masses (kDa) of the marker proteins.

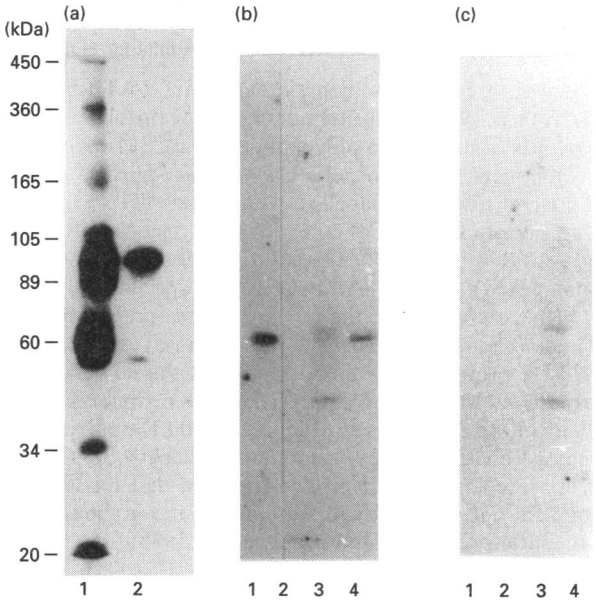

Figure 2 Identification of autophosphorylation activity of SR proteins

Heavy SR was extracted with EGTA to remove bound CaM and incubated in a phosphorylation assay as described in the Experimental section. (**a**) Autoradiogram of the phosphorylation pattern of SR proteins by the endogenous kinase with 50 μM [γ-^{32}P]ATP in the presence (lane 1) or absence (lane 2) of 0.6 μM CaM. In (**b**) and (**c**), subcellular fractions of rabbit skeletal muscle were separated by SDS/PAGE, transferred to nitrocellulose and allowed to renature as described by Celenza and Carlson (1986). After renaturation, autophosphorylation was assayed in the presence of either Ca^{2+}/CaM (**b**) or Ca^{2+} alone (**c**). The nitrocellulose was then dried and exposed for autoradiography: crude microsomes (lane 1); transverse tubules (lane 2); light SR (lane 3); heavy SR (lane 4).

absence (lane 2) of CaM. The major substrates of the CaM-dependent phosphorylation activity are polypeptides of 89, 60, 34 and 20 kDa. Polypeptides of 105, 165, 360 and 450 kDa were also phosphorylated in a CaM-dependent manner. Phosphorylation of the 97 and 53 kDa proteins occurred in a CaM-independent manner (lane 2).

In order to identify the polypeptide component of the SR membrane that was exhibiting the protein kinase activity, we adopted the method described by Celenza and Carlson (1986). This method detects autophosphorylation activity of protein kinases transferred to nitrocellulose after SDS/PAGE. This technique was modified to renature membrane proteins and to detect autophosphorylation activity in skeletal-muscle membrane fractions as described in the Experimental section. Nitrocellulose blots of various membrane fractions were incubated in a phosphorylation assay with [γ-^{32}P]ATP, and an autoradiogram is shown in Figures 2(b) and 2(c). A 60 kDa polypeptide in the microsomal fraction incorporates the greatest quantity of radiolabel in a Ca^{2+}/CaM-dependent manner (Figure 2b, lane 1). The 60 kDa autophosphorylated polypeptide was found to associate predominantly with the heavy-SR membranes (lane 4), although it was also present at low levels in the light-SR fraction (lane 3). No autophosphorylation of the 60 kDa polypeptide was detected in the absence of CaM (Figure 2c). In addition, CaM-independent autophosphorylation was also noted on polypeptides of 62 and 45 kDa enriched in the light-SR membranes (Figures 2b and 2c, lane 3). A similar autophosphorylation pattern for the 62 and 45 kDa polypeptides was observed in the presence of EGTA, whereas the 60 kDa polypeptide of the heavy SR did not show any autophosphorylation under these conditions (results not shown). No autophosphorylation activity was detected in the transverse-tubule-enriched fraction (lane 2).

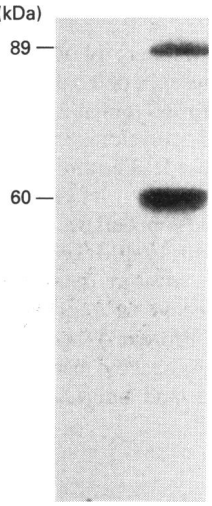

Figure 3 Photoaffinity labelling of the CaM-dependent protein kinase complex with [^{32}P]azido-ATP

The CaM-dependent protein kinase complex was isolated as described by Tuana and MacLennan (1988). Assay conditions were similar to those used for the phosphorylation reaction, except that 10 nM [^{32}P]azido-ATP replaced [γ-^{32}P]ATP in the kinase buffer. Photolabelling was carried out by irradiating for 2 min with u.v. light, and the reactions were terminated with SDS loading buffer and the samples were subjected to SDS/PAGE and autoradiography. Labelling with [^{32}P]azido-ATP in the presence (lane 1) and absence (lane 2) of excess unlabelled ATP is shown.

The 60 kDa polypeptide contains ATP- and CaM-binding sites

Protein kinases are known to contain high-affinity ATP-binding domains (Cohen, 1988). In an attempt to provide further evidence for the identity of the 60 kDa polypeptide as the CaM-dependent kinase component of the SR, the CaM-affinity-purified kinase fractions were covalently labelled with azido-ATP. Figure 3 shows an autoradiogram of the covalent incorporation of [^{32}P]azido-ATP in the presence (lane 1) and absence (lane 2) of excess unlabelled ATP. [^{32}P]Azido-ATP was specifically bound to a 60 kDa polypeptide in the purified kinase preparation. A significant amount of radiolabel incorporation was also observed in an 89 kDa protein which co-purified with the 60 kDa polypeptide on the CaM-affinity column. There was no incorporation of the label in the absence of u.v. light. The 60 kDa polypeptide is the major CaM-binding polypeptide in the heavy SR (Figure 1), and in a previous study it has been shown that this 60 kDa polypeptide also contained the CaM-binding sites in the isolated kinase preparation (Tuana and MacLennan, 1988).

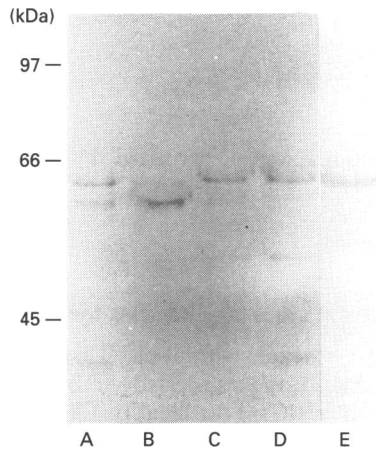

Figure 4 Immunostaining of various rabbit skeletal-muscle fractions with anti-brain CaM kinase II

After SDS/PAGE and blotting, the nitrocellulose was immunostained with anti-(brain CaM kinase II) antiserum by using alkaline-phosphatase-conjugated anti-rabbit IgG as described in the Experimental section. Lanes: A, crude microsomes; B, supernatant from pyrophosphate wash; C, pyrophosphate-washed heavy SR; D, isolated CaM kinase complex; E, junctional-face membrane.

The 60 kDa CaM kinase of SR and the soluble multifunctional CaM kinase II

Properties of the membrane-associated 60 kDa CaM kinase of the SR and the soluble multifunctional CaM-dependent kinase were compared. The immunoreactivity of an affinity-purified polyclonal antibody which recognizes conserved amino acid sequences of the multifunctional CaM kinases was examined in Western blots of subcellular fractions from skeletal muscle (Figure 4). Two immunoreactive polypeptides of 60 and 54 kDa were detected by the anti-(brain CaM kinase II) antiserum in crude microsomal preparations from skeletal muscle (lane A). The 54 kDa polypeptide could be selectively removed from the microsomal membranes with a pyrophosphate wash (lane B), which is thought to release cytoskeletal and peripheral proteins from the membrane preparations (Mitchell et al., 1983). The 60 kDa polypeptide remained associated with the membrane and was enriched in the heavy SR (lane C) and more specifically in

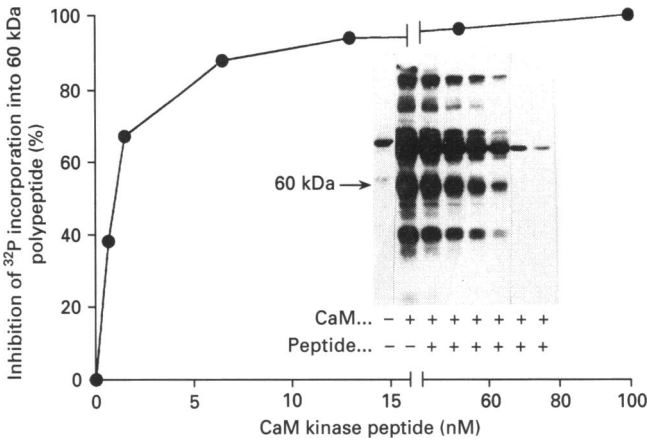

Figure 5 Inhibition of the CaM-dependent kinase complex by the CaM kinase II fragment (290–309)

SR membranes were phosphorylated (see the inset) as described in the absence (lane 1) or presence (lanes 2–8) of 0.6 μM CaM with increasing concentrations of the CaM kinase II fragment (290–309). The CaM-dependent phosphorylation of the 60 kDa polypeptide was identified by autoradiography and quantified by liquid-scintillation counting of dried gels. The inhibition of CaM-dependent ^{32}P incorporation into the 60 kDa polypeptide by the CaM kinase II fragment is expressed as a percentage of the control values measured in the presence of CaM and in the absence of CaM kinase II fragment.

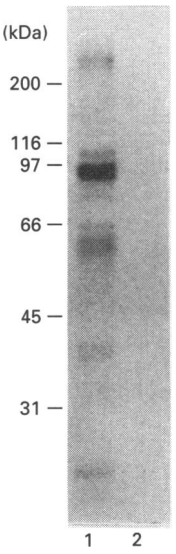

Figure 6 Immunoprecipitation of the SR CaM-dependent protein kinase complex by the anti-(brain CaM kinase II) antiserum

Rabbit skeletal-muscle SR membranes were phosphorylated and solubilized with NP40 as described in the Experimental section. The solubilized fractions were then incubated with the anti-(brain CaM kinase II) antibody, and the antibody complexes were isolated with Protein A–agarose. The proteins eluted from Protein A–agarose were subjected to SDS/PAGE and autoradiography. Immunoprecipitations with anti-(brain CaM kinase II) (lane 1) and with serum from non-immunized rabbits (lane 2) are shown.

the SR junctional-face membrane (lane E). It was also the major cross-reacting protein in the CaM-affinity-purified kinase preparation (lane D). This 60 kDa polypeptide migrated in SDS/PAGE with identical mobility with the 60 kDa polypeptide which exhibits CaM-dependent autophosphorylation activity.

By using a specific CaM antagonist, Wang and Best (1992) have provided strong evidence for the existence of a CaM kinase which is localized close to and is responsible for gating the Ca^{2+}-release channel. The sensitivity of the 60 kDa CaM kinase to this inhibitor of CaM functions was examined. This inhibitor acts primarily as a calmodulin antagonist and is a peptide of CaM kinase II, referred to as CaM kinase II inhibitory fragment (290–309) (Payne et al., 1988). Figure 5 (inset) shows the effects of increasing concentrations of CaM-kinase II fragment (290–309) on the CaM-dependent phosphorylation of SR proteins. The CaM-dependent phosphorylation was inhibited by the CaM kinase II fragment, and the inhibition of the CaM-dependent ^{32}P incorporation into the 450, 360, 165, 105, 89, 45, 34 and 20 kDa polypeptides followed the same qualitative pattern as that of the 60 kDa polypeptide. The CaM-dependent phosphorylation of the 60 kDa phosphoprotein was quantified by liquid-scintillation counting of the radioactive bands identified by autoradiography and cut from dried SDS gels. The inhibition of CaM-dependent ^{32}P incorporation into the 60 kDa polypeptide was plotted as a function of CaM kinase II fragment (290–309) concentration (Figure 5). CaM-dependent incorporation of ^{32}P into the 60 kDa polypeptide was completely eliminated at peptide concentrations of 100 nM. The estimated IC_{50} for this inhibition was approx. 2 nM, a value well below the 0.9–210 μM range of previously observed IC_{50} values for the brain multifunctional CaM kinases using CaM kinase II fragments of varying lengths (Colbran et al., 1989).

Associations of the 60 kDa CaM kinase of SR

In order to investigate the protein–protein interactions of the 60 kDa kinase of the SR membrane, immunoprecipitation experiments were performed. SR membranes were incubated with [γ-^{32}P]ATP under phosphorylating conditions and solubilized with NP40. The solubilized proteins were immunoprecipitated and analysed by SDS/PAGE and autoradiography. Figure 6 represents an autoradiogram showing that a number of phosphoproteins, including the 60 kDa polypeptide, were immunoprecipitated by the anti-(brain CaM kinase II) antiserum (lane 1). The other phosphoproteins that were co-immunoprecipitated included the 89, 34, 20, 105 and 360 kDa phosphoproteins. None of the phosphoproteins were immunoprecipitated by serum from non-immunized rabbits (lane 2). These phosphoproteins were also found to co-migrate on 5–20% linear sucrose-density gradients (results not shown), further suggesting an association among these polypeptides.

Polyclonal antibodies to the gel-purified 60 kDa kinase polypeptide were raised in guinea pigs in order to characterize this SR protein further. Western blots were performed on the material isolated from the CaM-affinity column (Figure 7). The anti-(60 kDa kinase polypeptide) serum cross-reacts with a single 60 kDa polypeptide (lane 2), whereas the other components of the SR CaM kinase complex were not immunoreactive. The anti-(60 kDa polypeptide) serum did not cross-react with the CaM kinase isolated from cardiac SR (lane 1), which consists of polypeptides of 120, 70, 60, 54 and 34 kDa (B. S. Tuana, unpublished work) or the soluble multifunctional CaM kinase II from skeletal muscle and brain (results not shown). The purified SR CaM kinase complex was phosphorylated as described above, treated with SDS and immunoprecipitated with the anti-(60 kDa polypeptide) serum. Only the 60 kDa phosphoprotein was immunoprecipitated by the anti-(SR 60 kDa polypeptide) serum from the CaM-dependent phosphorylation reaction (results not shown). Since the anti-(60 kDa polypeptide) serum only recognized the SDS-denatured form of the 60 kDa polypeptide,

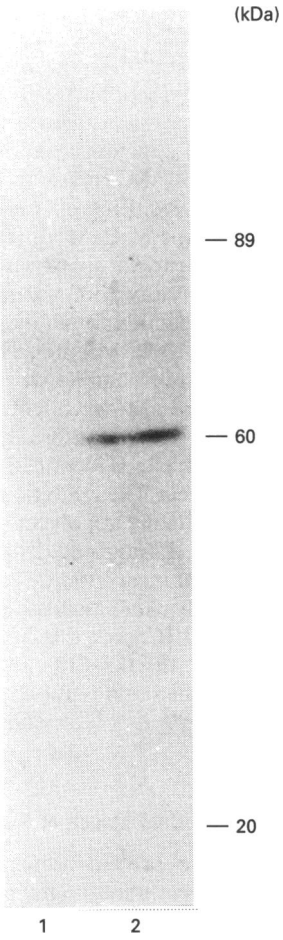

Figure 7 Immunoreactivity of the anti-(60 kDa polypeptide) serum with components of the CaM kinase complex

The 60 kDa kinase was isolated from a CaM affinity column and gel purified. Polyclonal antibodies against the 60 kDa polypeptide were raised in guinea pigs as described in the Experimental section. A Western blot was performed on the material isolated on the CaM affinity column from cardiac SR (lane 1) and skeletal-muscle SR (lane 2). A 1/250 dilution of anti-(60 kDa polypeptide) serum was used, and the reaction was developed with alkaline-phosphatase-conjugated second antibody.

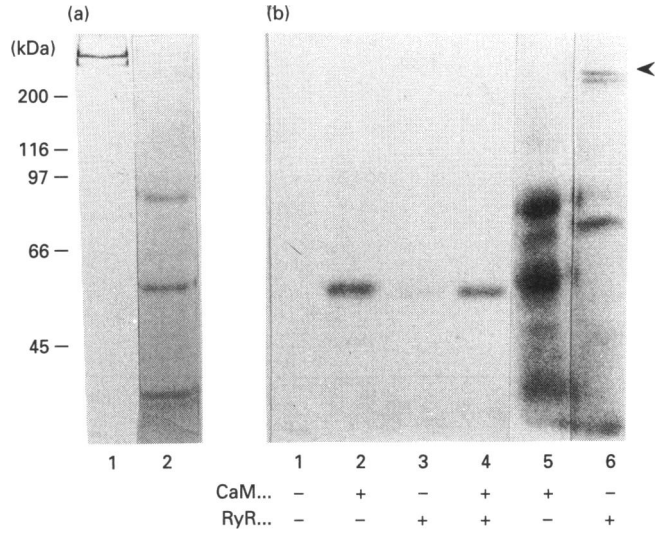

Figure 8 CaM-dependent phosphorylation of the SR Ca^{2+}-release channel

The purified ryanodine receptor and the CaM kinase complex were separated by SDS/PAGE, and the protein composition was revealed by silver staining (panel **a**, lanes 1 and 2 respectively). The purified ryanodine receptor and CaM kinase complex were reconstituted in a phosphorylation reaction, and the phosphoprotein composition was analysed by SDS/PAGE and autoradiography. Panel (**b**) represents a typical autoradiogram of CaM-dependent phosphorylation of purified CaM kinase (lanes 1 and 2), purified ryanodine receptor plus kinase complex (lanes 3 and 4), heat-denatured SR plus purified kinase complex (lane 5), and purified ryanodine receptor plus purified protein kinase C (lane 6). The arrow-head indicates the phosphorylated ryanodine receptor.

none of the phosphoproteins could be immunoprecipitated in the absence of SDS.

The CaM kinase complex and ryanodine-receptor phosphorylation

Since CaM-dependent phosphorylation has been implicated in the regulation of Ca^{2+} release from the SR, we sought to resolve whether the ryanodine receptor was a substrate for the CaM-dependent protein kinase of SR. Figure 8(a) is a representative silver-stained SDS gel of the purified ryanodine receptor and the CaM-dependent protein kinase complex of skeletal-muscle SR. The purified ryanodine receptor migrated as a doublet of 450 and 425 kDa (lane 1), whereas the CaM kinase complex appeared as polypeptides of 89, 60 and 34 kDa (the 20 kDa species was run off the gel) (lane 2). The ryanodine receptor (1 μg) was incubated with the CaM kinase in the presence of [γ-^{32}P]ATP and phosphorylation buffer as described above. The resulting autoradiogram is shown in Figure 8(b). Despite CaM-dependent incorporation of radiolabel into the 60 kDa CaM kinase (lane 2) and of its substrates (results not shown), no phosphorylation could be detected at the level of the ryanodine receptor (lane 4) by using three different preparations of the purified ryanodine receptor and the CaM kinase. In heat-inactivated preparations of the SR membrane, the purified kinase phosphorylated the 89, 60, 34 and 20 kDa polypeptides, but not the ryanodine receptor (lane 5), although the ryanodine receptor was a substrate of purified protein kinase C (lane 6), which appears as an 80 kDa phosphoprotein. The phosphoprotein located close to the dye front (lane 6) may be a breakdown product of the Ca^{2+}-release channel or protein kinase C.

DISCUSSION

A CaM-dependent protein kinase activity has been shown to associate with SR membranes of skeletal muscle and phosphorylate polypeptides of 450, 360, 165, 105, 89, 60, 34 and 20 kDa (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1982; Tuana and MacLennan, 1984; Seiler et al., 1984). As it remained unclear as to which polypeptide of the SR was a CaM kinase, we used a variety of techniques, including the renaturation of autophosphorylation activity of protein kinases separated in SDS/PAGE and transferred to nitrocellulose (Celenza and Carlson, 1986), to elucidate the identity of this kinase. In this study, we have identified the 60 kDa polypeptide of skeletal-muscle SR as the CaM-dependent protein kinase that undergoes CaM-dependent autophosphorylation and associates with substrate proteins of 360, 105, 89, 34 and 20 kDa. Our results, showing that the 60 kDa polypeptide contains CaM- and ATP-binding sites, as well as its ability to undergo CaM-dependent autophosphorylation in renaturation assays on nitrocellulose, provide strong evidence that this polypeptide is the CaM-

dependent protein kinase of the SR membrane. Using the renaturation-overlay technique, we have also identified a Ca^{2+}/CaM-independent autophosphorylation activity associated with polypeptides of 62 and 45 kDa enriched in the light-SR membranes. Since a Ca^{2+}-independent kinase activity has been described in SR vesicles that phosphorylates polypeptides of 62, 42 and 20 kDa (Campbell and Shamoo, 1980), our data suggest that this kinase activity may be attributable to the 62 and 45 kDa polypeptides of the light SR. Although the 60 kDa CaM kinase was found to be enriched in the heavy SR, we did not detect any autophosphorylation activity in the transverse-tubule membranes, although new CaM-binding polypeptides of 165, 100 and 80 kDa were identified and found to be enriched in these membranes. In addition, the blot-overlay technique also allowed the detection of a 33 kDa polypeptide that bound CaM in the presence of EGTA and in the apparent absence of Ca^{2+}.

Although our data provide clear evidence for a 60 kDa polypeptide as a CaM-dependent protein kinase, we cannot rule out the existence of other polypeptides exhibiting CaM-dependent protein kinase activity, since additional CaM-binding and ATP-binding polypeptides were detected in the SR membrane, and their enzyme activities may not be fully renatured under our assay conditions.

The 60 kDa CaM kinase of SR is different from the soluble multifunctional CaM kinase II, since it is membrane-associated and can only be solubilized with detergents. Affinity-purified polyclonal antibodies raised against the brain multifunctional CaM kinase II subunits cross-react with the 60 kDa polypeptide of SR as well as with a 54 kDa polypeptide in skeletal muscle. However, this is likely, since these antibodies were raised against a peptide sequence that is highly conserved in different CaM-dependent protein kinases. Our results indicate that there is a selective association of the 60 kDa CaM kinase with specific membrane populations within the muscle cell, since a 54 kDa polypeptide recognized by the antibodies was readily dissociated from the microsomal membranes by a pyrophosphate wash, known to remove peripheral proteins (Mitchell et al., 1983). In contrast, the 60 kDa CaM kinase was not only enriched in the heavy-SR fractions but closely associated with the junctional-face membrane of the terminal cisternae. Unlike the soluble multifunctional CaM-dependent protein kinases, which appear to form homogeneous complexes of α and β subunits (Woodgett et al., 1983; Schulman and Lou, 1989; Kanaseki et al., 1991), the 60 kDa CaM-dependent protein kinase of skeletal-muscle SR forms a heterogeneous complex with phosphoproteins of 360, 105, 89, 34 and 20 kDa. Anti-(60 kDa polypeptide) antibodies did not cross-react with the other phosphoproteins, suggesting that the 360, 105, 89, 34 and 20 kDa proteins do not share epitopes that are in common with the 60 kDa polypeptide. This is consistent with a previous study which showed that the 89, 60, 34 and 20 kDa proteins of the CaM kinase complex were structurally distinct polypeptides (Tuana and MacLennan, 1988). The anti-(60 kDa SR kinase) antiserum did not cross-react with a CaM kinase from cardiac SR or with the subunits of the brain multifunctional CaM kinase II. Furthermore, the 60 kDa CaM kinase of SR was much more sensitive to an inhibitor of CaM functions than was the soluble brain CaM kinase II. These results suggest that the 60 kDa polypeptide of SR is a distinct isoenzyme of the CaM-dependent protein kinase family.

Protein phosphorylation is known to modulate the activity of various types of ion channels (Levitan, 1988; Catterall, 1991; Dolphin, 1991). CaM-dependent protein kinases have been implicated in regulating Cl^- channels (Nishimoto et al., 1991), enhancing DHP-sensitive Ca^{2+} current in smooth muscle (McCarron et al., 1992), potentiating Ca^{2+} transients (Meyer et al., 1992) and increasing the open probability of the Ca^{2+}-release channel of cardiac SR (Witcher et al., 1991). In considering a physiological function of the CaM kinase complex of skeletal-muscle SR, its specific association with the junctional-face membrane argues for an important role in excitation–contraction coupling, since both the ryanodine and DHP receptors would be in close vicinity (Block et al., 1988). Wang and Best (1992) have identified a reversible phosphorylation event which was involved in the inactivation of the Ca^{2+}-release channel current in its native lipid environment and inhibitable by the CaM kinase II fragment (273–302). Our results suggest that the protein kinase involved in channel inactivation is probably the 60 kDa CaM kinase of the SR, since it was potently inhibited by the CaM kinase II inhibitory fragment which mimics the CaM-binding domain of the enzyme and inhibits its activity (Payne et al., 1988). We have also observed significant levels of CaM-dependent phosphorylation of a 450 kDa polypeptide in rabbit skeletal-muscle SR membranes. Some doubt exists as to the exact identity of this high-molecular-mass phosphoprotein in skeletal muscle (Seiler et al., 1984), which is either the ryanodine receptor (Chu et al., 1990) or an unrelated polypeptide (Witcher et al., 1991). The cardiac and brain ryanodine receptors were substrates of the soluble CaM kinase II, and phosphorylation occurred at a mutually conserved site, which was altered in the skeletal-muscle ryanodine receptor (Witcher et al., 1991, 1992). Previous inability to record substantial CaM-dependent phosphorylation of the skeletal-muscle ryanodine receptor may be attributable to the use of the brain CaM kinase II to generate CaM-dependent phosphorylation, which is different in substrate specificity and time-course from the endogenous SR CaM kinase (Tuana and MacLennan, 1988). In reconstitution experiments of the purified ryanodine receptor and CaM kinase of SR, we were unable to detect any CaM-dependent phosphorylation of the ryanodine receptor, although it served as a substrate for protein kinase C (Takasago et al., 1991). Furthermore, in heat-inactivated SR membranes, the ryanodine receptor did not serve as a substrate for the purified 60 kDa kinase, suggesting that CaM or any associated protein was not masking potential CaM-dependent phosphorylation sites. Since the ryanodine receptor is not phosphorylated by the 60 kDa CaM kinase, we propose a mechanism in which the phosphorylation of CaM kinase and/or of its substrates in skeletal-muscle SR could lead to the observed inactivation of the Ca^{2+}-release current (Wang and Best, 1992) through direct steric hindrance. The protein–protein interactions required in such a mechanism are supported by the estimated stoichiometric ratio of 1:1 to 1:2 between the 60 kDa CaM kinase and the Ca^{2+}-release channel (Meissner, 1974; Campbell and MacLennan, 1982; Lai et al., 1988), and the CaM-dependent phosphorylation activity of 1–2.5 nmol of phosphate incorporated/mg of 60 kDa polypeptide is comparable with the activity of other SR kinases (Orr et al., 1991). The steric hindrance of the Ca^{2+}-release channel by the 360, 105, 89, 60, 34 and 20 kDa phosphoproteins of the CaM kinase complex could occur in a manner that is qualitatively similar to the phosphorylation-dependent interaction that occurs between phospholamban and the Ca^{2+}-ATPase in cardiac SR (Tada et al., 1988; Fujii et al., 1989; James et al., 1989). Alternatively, the ryanodine receptor could be sensitive to changes in surface charge (Shoshan-Barmatz, 1988; Gechtman et al., 1991) generated by the CaM-dependent phosphorylation of the CaM kinase substrates. The dephosphorylation event that would be required to reset the system could be generated from a phosphatase activity which is targeted to the SR (Horl and Heilmeyer, 1978; Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983; Wegener et al., 1984; Hubbard et al., 1990). In this regard, the

Ca^{2+}-activated K^+ channel has been shown to be modulated in a similar fashion by a membrane-associated kinase/phosphatase regulatory complex (Chung et al., 1991). The Ca^{2+}/CaM-independent autophosphorylation activity of the 62 and 45 kDa polypeptides found in the light SR may play a role in Ca^{2+} transport, since a high density of the Ca^{2+}-ATPase molecules is localized in this fraction. The membrane-associated 33 kDa CaM-binding polypeptide may play a role in sequestering free CaM to the regions of triad junction under resting conditions (low [Ca^{2+}]), by analogy to the way neuromodulin is thought to serve as a sink for CaM in neuronal membranes (Liu and Storm, 1990). Now that we have determined the identity of the CaM kinase and the Ca^{2+}/CaM-independent kinase of skeletal-muscle SR, further studies are underway to define the precise mechanisms by which these membrane-associated kinases may regulate SR function.

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