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Affinity Labeling of Calmodulin-binding Proteins in Skeletal Muscle Sarcoplasmic Reticulum*

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^{125}I-Calmodulin (^{125}I-CaM) binding to sarcoplasmic reticulum (SR) membranes isolated from skeletal muscle cells was investigated, and the CaM receptors associated with the membrane were identified by using the photoaffinity cross-linker methyl-4-azidobenzimidate or the chemical cross-linker dithiobis-*N*-hydroxysuccinimidyl propionate. Exogenous CaM binds to CaM-depleted membranes in a Ca^{2+}- or Mg^{2+}-dependent way. When both cations are added together to the reaction medium, the stimulatory effects appear to be additive, suggesting that Ca^{2+} and Mg^{2+} act by two distinct mechanisms. The Ca^{2+}/Mg^{2+}-dependent binding of CaM is specific since it is inhibited by unlabeled CaM or by trifluoperazine. Furthermore, it is saturable and shows one class of high affinity binding sites with a K_D of about 52 nM and a β_{max} of about 5 pmol/mg of protein. The sensitivity of Ca^{2+} is expressed in two steps reaching half-saturation at free Ca^{2+} concentrations of about 1.6×10^{-7} and 3×10^{-5} M, respectively. On the other hand, the sensitivity to Mg^{2+} is expressed in one step with a half-saturation Mg^{2+} concentration of about 2×10^{-3} M. Electrophoretic analysis in a polyacrylamide gradient and subsequent autoradiography demonstrated a major CaM-binding protein of about 60 kDa and five minor CaM receptors of about 148, 125, 41, 33, and 23 kDa, respectively. The major labeled protein (60 kDa) probably represents the CaM-dependent component involved in Ca^{2+} release from SR, whereas the others represent a previously unrecognized class of CaM receptors in skeletal SR.

The Ca^{2+} concentration within the cells is maintained by the Ca^{2+} pump mechanisms of the plasma membrane and of the endoplasmic reticulum. The activity of these systems is particularly important in muscle cells to determine the contraction-relaxation cycle (1–3).

In plasma membranes, calmodulin (CaM)[1] regulates Ca^{2+} transport by interacting directly with the Ca^{2+}-ATPase (4), whereas in cardiac sarcoplasmic reticulum (SR), the Ca^{2+} pump is indirectly regulated through the CaM-dependent phosphorylation of phospholamban (5–10).

SR membranes isolated from skeletal muscle cells do not contain phospholamban, and no interaction of CaM with the ATPase enzyme was observed. On the other hand, some effects of CaM antagonists on Ca^{2+} transport by skeletal SR have been described, but no conclusive involvement of CaM was found in this process (11–16). Nevertheless, the presence of CaM in skeletal SR has been well documented by several investigators (11, 12, 17–20), and the presence of an endogenous CaM-dependent kinase activity has been reported in these membranes (11, 12, 21, 22).

Campbell and MacLennan (12) observed that CaM stimulates phosphorylation of an $M_r = 60,000$ protein which appears to be involved in the process of Ca^{2+} release from SR. These findings were supported by those of Kim and Ikemoto (23), who observed a good correlation between the amount of P_i incorporation into the $M_r = 60,000$ protein and the extent of inhibition of Ca^{2+} release. However, Meissner (18) observed that CaM inhibits Ca^{2+} release even in the absence of ATP, which suggests that CaM may regulate Ca^{2+} release by direct interaction with the Ca^{2+} channel rather than with a kinase enzyme.

Although several proteins of skeletal SR appear to be phosphorylated in a CaM-dependent way (11, 12, 21–23), it has been difficult to visualize CaM-binding proteins in this system.

In this work, I studied the CaM binding properties of the skeletal SR, and I identified some protein components of the membranes which interact specifically with CaM. The possible identity of these CaM receptors is discussed.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-labeled Bolton-Hunter reagent and [^{125}I]iodine were purchased from Amersham Corp. Dithiobis-*N*-hydroxysuccinimidyl propionate (Lomant's reagent), methyl-4-azidobenzoimidate, and 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (IODO-GEN) were obtained from Pierce Chemical Co. Molecular mass markers were purchased from Sigma.

Preparation of Calmodulin-depleted Sarcoplasmic Reticulum Membranes—SR was isolated from rabbit white skeletal muscle as previously described (24). Then the membranes were washed twice in a solution containing 50 mM KCl, 10 mM Tris-HCl (pH 7.0), 1.5 mM EDTA, and 20 μM phenylmethylsulfonyl fluoride. After centrifugation for 30 min at 40,000 × *g*, the pellets were washed once in 50 mM KCl, 10 mM Tris maleate (pH 7.0), and 20 μM phenylmethylsulfonyl fluoride. Finally, the membranes were resuspended in the same medium and immediately utilized in the experimental assays.

The protein was determined by the biuret method using bovine serum albumin as standard (25).

Preparation of ^{125}I-Calmodulin—CaM isolated from bovine brain (26) was radiolabeled with ^{125}I-labeled Bolton-Hunter reagent (27) essentially according to the method of Agre *et al.* (28). The reagent (500 μCi of a solution in benzene (2200 Ci/mmol)) was dried with N_2 stream. Then 200 μg of CaM in 40 mM $NaPO_4$ (pH 8.1) were added to the residue and incubated for 60 min at 0 °C. The reaction was stopped by diluting with 0.5 ml of a solution containing 0.5 mg/ml gelatin in 100 mM Hepes (pH 6.5), 1 mM NaN_3, 0.2 mM dithiothreitol,

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[1] The abbreviations used are: CaM, calmodulin; SR, sarcoplasmic reticulum; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

and 50 μM $CaCl_2$. Finally, the mixture was dialyzed overnight at 2 °C against the same buffer.

For experiments of photoaffinity labeling of the membranes, pure CaM was iodinated by the IODO-GEN method (29), and the derivative, azido-CaM, was obtained by using methyl-4-azidobenzimidate as described previously (30).

Chemical Cross-linking of ^{125}I-Calmodulin to Sarcoplasmic Reticulum Membranes—CaM-depleted SR (~0.3 mg) was incubated for 30 min at 25 °C in medium (400 μl) containing 100 mM Hepes (pH 7.0), 130 mM KCl, 1 mM EGTA (if present), and the concentrations of $CaCl_2$, $MgCl_2$, and ^{125}I-CaM (20,000 cpm/pmol) indicated in the figure legends. The cross-linker dithiobis-*N*-hydroxysuccinimidyl propionate (31) was added to about 0.15 mg/ml; and 1h later, the cross-linking was quenched by addition of glycine (to 0.6 mM) (28). Bound and free ^{125}I-CaMs were separated by centrifugation for 3 min in an Eppendorf centrifuge. The pellets were washed three times with 1.5 ml of a solution containing 100 mM Hepes (pH 7.0), 130 mM KCl, 50 μM $CaCl_2$, and 500 μM $MgCl_2$; and finally, they were counted in a γ-counter.

Ca^{2+}- and Mg^{2+}-independent binding was measured by including EDTA (10 mM) in reaction medium without Ca^{2+}.

CaM-binding proteins were identified by autoradiography after electrophoretic separation of the proteins in a 7–15% polyacrylamide gradient according to the Laemmli method (32). Molecular mass markers (bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14 kDa; and myosin, 200 kDa) were used to estimate molecular masses of the sample proteins. The gels were stained with Coomassie Blue; and after drying, they were exposed at −70 °C for 1 week on Du Pont-New England Nuclear Cronex two-dimensional x-ray film using a High Plus intensifying screen from the same manufacturer.

Photoaffinity Cross-linking of ^{125}I-Calmodulin to Sarcoplasmic Reticulum Membranes—CaM-depleted membranes were incubated in the dark for 30 min at 25 °C in medium containing 25 mM Hepes (pH 7.2), 130 mM KCl, 10 mM $MgCl_2$, 500 μM $CaCl_2$, and 100 nM azido-^{125}I-CaM. Then, the membranes were photolyzed for 8 min with an ultraviolet lamp type UVS-11; and finally, they were centrifuged and washed for radioactivity analysis as described above.

RESULTS

Effect of Cations on ^{125}I-Calmodulin Binding to Sarcoplasmic Reticulum Membranes—CaM interacts with SR in a cation-dependent way. Fig. 1 shows that either Ca^{2+} or Mg^{2+} stimulates the binding of CaM to the membranes and that the effect of Mg^{2+} is even more potent than that of Ca^{2+}. About 2 pmol are bound per mg of reticulum protein in the absence of cations (EDTA present), whereas binding of about 4 and 5

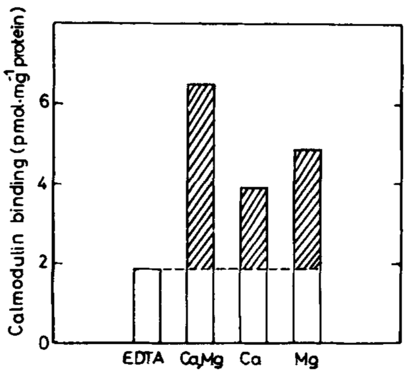

FIG. 1. **Effect of Ca^{2+} and Mg^{2+} on interaction of calmodulin with sarcoplasmic reticulum membranes.** CaM-depleted SR (0.3 mg) was incubated for 30 min at 25 °C in medium containing 100 mM Hepes (pH 7.0), 130 mM KCl, and 100 nM ^{125}I-CaM (20,000 cpm/pmol) in the presence of 10 mM EDTA (*EDTA*), 0.7 mM $CaCl_2$ and 10 mM $MgCl_2$ (*Ca,Mg*), 0.7 mM $CaCl_2$ (*Ca*), or 10 mM $MgCl_2$ and 1 mM EGTA (*Mg*). Cross-linking and separation of bound and free ^{125}I-CaMs were performed as described under "Experimental Procedures." The *open bars* represent cation-independent CaM binding, whereas the *hatched bars* represent cation-dependent CaM binding to SR.

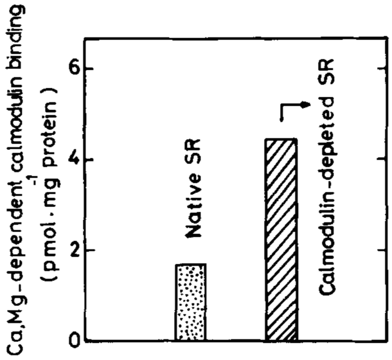

FIG. 2. **Ca^{2+}/Mg^{2+}-dependent binding of calmodulin to native sarcoplasmic reticulum and to EDTA-treated SR.** Native SR or EDTA-treated membranes were incubated with ^{125}I-CaM (100 nM) in the presence of 0.7 mM $CaCl_2$ and 10 mM $MgCl_2$ as described for Fig. 1.

pmol/mg of protein occurs in the presence of Ca^{2+} and Mg^{2+}, respectively. When both cations are added together to the reaction medium, the stimulatory effect observed is additive, which suggests that Ca^{2+} and Mg^{2+} act by two distinct mechanisms.

The magnitude of the Ca^{2+}/Mg^{2+}-dependent binding to SR depends on the previous washing of the membranes with EDTA (Fig. 2). The EDTA-treated membranes bind higher amounts of CaM (4.5 pmol/mg of protein) as compared to those bound by native membranes (1.7 pmol/mg of protein). It appears that SR contains endogenous CaM which must be removed from the membranes to become the receptors available to exogenous CaM.

Analysis of Affinity and Capacity of Sarcoplasmic Reticulum to Bind Calmodulin—Fig. 3 shows that the binding of CaM to SR membranes increases as the CaM concentration increases in the medium up to about 200 nM. However, if the binding curve, measured in the absence of divalent cations (nonspecific binding), is subtracted from the total binding curve, Ca^{2+}/Mg^{2+}-dependent binding is obtained which is saturable at about 100 nM CaM (Fig. 3*A*). Scatchard analysis shows that there is essentially one class of binding sites which binds CaM in a Ca^{2+}/Mg^{2+}-dependent manner (Fig. 3*B*). The value calculated for half-saturation (K_D) was about 52 nM and that for maximal binding (β_{max}) was 5 pmol/mg of protein.

Analysis of Specificity of Sarcoplasmic Reticulum to Bind Calmodulin—In order to investigate the specificity of the cation-dependent binding of CaM to SR membranes, experiments were carried out in the presence of trifluoperazine or unlabeled CaM.

Fig. 4 shows that trifluoperazine competitively inhibits the interaction of CaM with its targets. Binding of about 5 pmol/mg of protein was inhibited 50% by a 50 μM concentration of the drug (I_{50}). This value is in agreement with other effects previously observed in SR membranes (13, 16) which indicate that a large partitioning of the drug into the membrane is required to obtain phenothiazine effects.

Similarly, unlabeled CaM competed for the binding of ^{125}I-CaM with an I_{50} of about 380 nM (Fig. 5). These results indicate that Ca^{2+}/Mg^{2+}-dependent binding of CaM is specific. In contrast, the cation-independent binding is nonspecific since no significant competitive effects were observed in the presence of trifluoperazine or unlabeled CaM (data not shown).

Characterization of Effect of Ca^{2+} and Mg^{2+} on Calmodulin Binding to Sarcoplasmic Reticulum Membranes—CaM binding to SR depends on the concentration of calcium in the reaction medium (Fig. 6). Either in the presence or absence

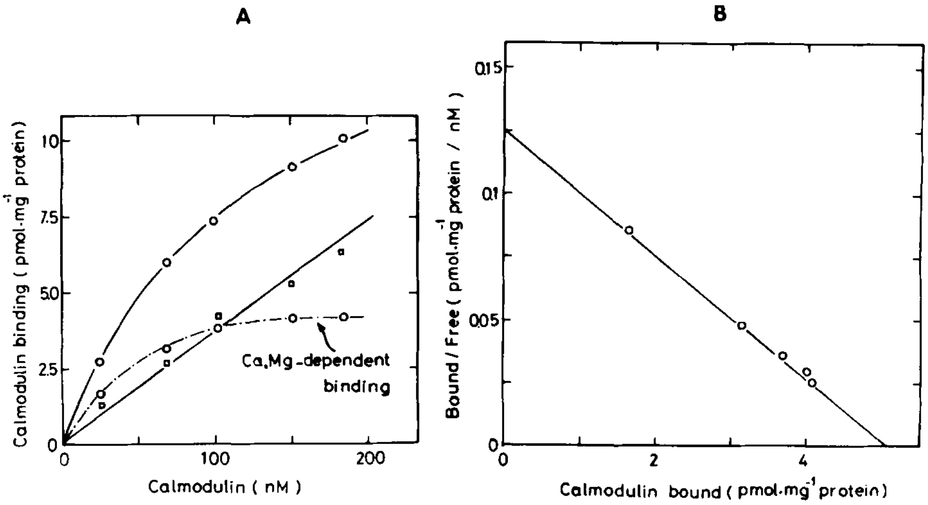

FIG. 3. **Calmodulin binding to sarcoplasmic reticulum as function of calmodulin concentration.** CaM-depleted SR was incubated with various concentrations of ^{125}I-CaM in the presence of 10 mM EDTA or 0.7 mM $CaCl_2$ and 10 mM $MgCl_2$ as described under "Experimental Procedures." *A*, total CaM binding (O——O), cation-independent CaM binding (□——□), and Ca^{2+}/Mg^{2+}-dependent CaM binding (O—·—O). *B*, Scatchard analysis of Ca^{2+}/Mg^{2+}-dependent CaM binding to SR.

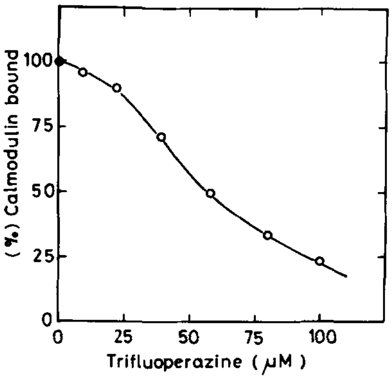

FIG. 4. **Effect of trifluoperazine on Ca^{2+}/Mg^{2+}-dependent calmodulin binding to sarcoplasmic reticulum.** CaM-depleted SR was incubated with 100 nM ^{125}I-CaM in the presence of 0.7 mM $CaCl_2$, 10 mM $MgCl_2$, and various concentrations of trifluoperazine as described under "Experimental Procedures."

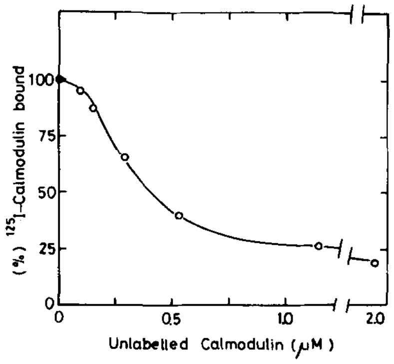

FIG. 5. **Competition of unlabeled calmodulin for Ca^{2+}/Mg^{2+}-dependent ^{125}I-calmodulin binding to sarcoplasmic reticulum.** CaM-depleted SR was incubated with 100 nM ^{125}I-CaM in the presence of 0.7 mM $CaCl_2$, 10 mM $MgCl_2$, and various concentrations of unlabeled CaM as described under "Experimental Procedures."

of Mg^{2+}, the sensitivity to Ca^{2+} is expressed in two steps. The first one has a half-saturation free Ca^{2+} concentration ($K_{m(\mathrm{Ca})}$) of about 1.6×10^{-7} M, whereas the second one has a $K_{m(\mathrm{Ca})}$ value of about 3×10^{-5} M.

At all concentrations of Ca^{2+} studied, the effect of Mg^{2+} appears to be additive to that of Ca^{2+}, in agreement with the results depicted in Fig. 1. At pCa 4, about 2 pmol of CaM/mg of protein are bound in the absence of Mg^{2+}, whereas in its

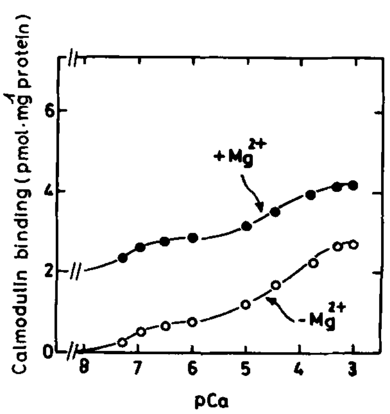

FIG. 6. **Ca^{2+} dependence of ^{125}I-calmodulin binding to sarcoplasmic reticulum.** CaM-depleted SR was incubated with 100 nM ^{125}I-CaM at various pCa values in the absence of $MgCl_2$ (O) or in the presence of 10 mM $MgCl_2$ (●) as described under "Experimental Procedures." The values plotted in the graph represent specific binding obtained after subtracting the binding of calmodulin in the absence of cations (EDTA present).

presence, binding of about 4 pmol/mg of protein is observed.

The biphasic sensitivity to Ca^{2+} indicates that SR contains two types of CaM receptors which require different Ca^{2+} concentrations to bind CaM in the range of high affinity. In contrast, the effect of Mg^{2+} shows only a plateau requiring Mg^{2+} concentrations of about 2×10^{-3} M for half-maximal saturation (Fig. 7). The sensitivity to Mg^{2+} was studied in the presence of Ca^{2+} concentrations which permit maximal Ca^{2+}-dependent binding of CaM (~2 pmol/mg of protein).

Detection of Calmodulin-binding Proteins in Sarcoplasmic Reticulum Membranes—The CaM receptors of SR were visualized by covalent cross-linking to ^{125}I-CaM and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiography.

Fig. 8 shows CaM-protein complexes formed by photoaffinity cross-linking. About six ^{125}I-containing products can be distinguished in the autoradiograph (Fig. 8*B, lane 4'*). Their molecular masses are about 165, 142, 77, 58, 50, and 40 kDa, which correspond to complexes between ^{125}I-CaM (17 kDa) and SR proteins of about 148, 125, 60, 41, 33, and 23 kDa, respectively. At the top of the gel, we can visualize a radioactive band ($M_r > 200{,}000$), in agreement with previous observations by Seiler *et al.* (22) in vesicles of junctional SR. The large radioactive band at the bottom of the gel represents

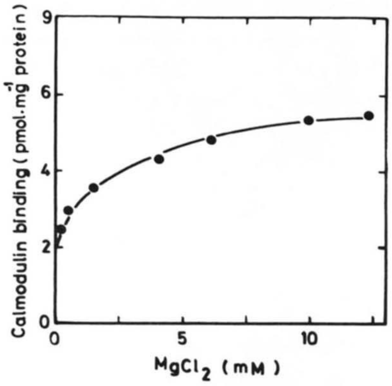

FIG. 7. **Mg^{2+} dependence of ^{125}I-calmodulin binding to sarcoplasmic reticulum.** CaM-depleted SR was incubated with 100 nM ^{125}I-CaM in the presence of 0.7 mM $CaCl_2$ and various concentrations of $MgCl_2$ as described under "Experimental Procedures." The graph represents specific binding of calmodulin as described for Fig. 6.

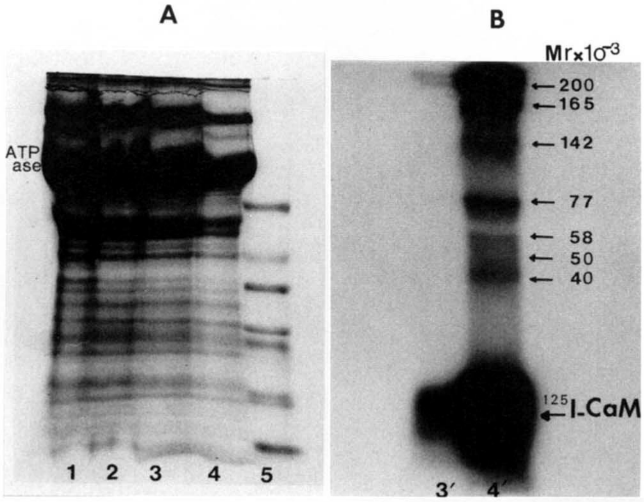

FIG. 8. **Photoaffinity cross-linking of ^{125}I-calmodulin to sarcoplasmic reticulum membranes.** CaM-depleted SR (0.5 mg) was incubated and photoaffinity-labeled with 100 nM ^{125}I-CaM in the presence of 10 mM EDTA or 0.5 mM $CaCl_2$ and 10 mM $MgCl_2$ as described under "Experimental Procedures." Then, about 200 μg of the SR protein were used for gel electrophoresis according to the Laemmli method (32). *A*, Coomassie Blue staining patterns of SR proteins: native SR (*lane 1*), EDTA-washed SR (*lane 2*), SR after cross-linking in the presence of EDTA (*lane 3*), SR after cross-linking in the presence of Ca^{2+} and Mg^{2+} (*lane 4*), and molecular weight markers (*lane 5*). *B*, autoradiograph showing photoaffinity-cross-linked ^{125}I-CaM to SR proteins: labeling in the presence of EDTA (*lane 3′*) and labeling in the presence of Ca^{2+} and Mg^{2+} (*lane 4′*).

free ^{125}I-CAM (17 kDa) which was not completely removed during the wash step.

The labeling of SR proteins with the photoaffinity probe azido-^{125}I-CaM is dependent on the presence of Ca^{2+} and Mg^{2+} since no incorporation of the probe was observed in the absence of cations (Fig. 8*B*, *lane 3′*). Furthermore, the efficiency of cation-dependent CaM binding to SR proteins is relatively low since the altered mobility of the CaM-protein complexes was not visualized in the Coomassie Blue staining protein pattern (Fig. 8*A*).

CaM-binding proteins of skeletal SR were also detected by chemical cross-linking using dithiobis-*N*-hydroxysuccinimidyl propionate (Fig. 9). In the presence of Ca^{2+} and Mg^{2+}, the autoradiograph shows a labeled protein pattern (*lane 5*) similar to that obtained by photoaffinity cross-linking (Fig. 8*B*, *lane 4′*). However, an additional ^{125}I-containing product (M_r

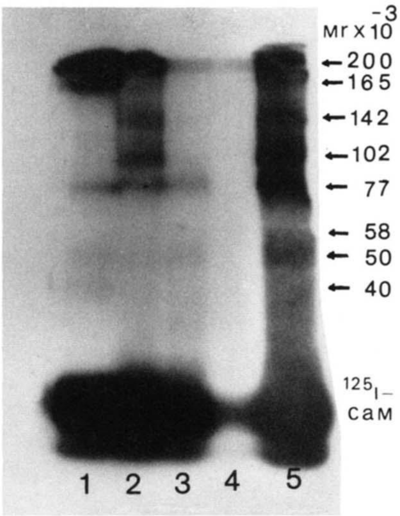

FIG. 9. **Chemical cross-linking of ^{125}I-calmodulin to sarcoplasmic reticulum membranes.** CaM-depleted SR was incubated and affinity-labeled with 100 nM ^{125}I-CaM in the presence of 0.7 mM $CaCl_2$ and 10 mM $MgCl_2$ (*lane 5*), 10 mM EDTA (*lane 4*), 0.7 mM $CaCl_2$ (*lane 3*), or 10 mM $MgCl_2$ and 1 mM EGTA (*lane 2*) as described under "Experimental Procedures." *Lane 1* represents native SR membranes labeled with ^{125}I-CaM in the presence of 0.7 mM $CaCl_2$ and 10 mM $MgCl_2$. About 150 μg of SR protein were used in the gel electrophoresis.

~102,000), which may represent a complex between CaM and a 85-kDa protein, is observed when labeling was performed in the presence of Mg^{2+} (Fig. 9, *lanes 2* and *5*). The 85-kDa component as well as the 125-kDa component (*lanes 2* and *5*) require Mg^{2+} for CaM binding since they are not visualized when only Ca^{2+} exists in the reaction medium (*lane 3*). In contrast, the 60-kDa protein interacts with CaM in the presence of either Ca^{2+} (*lane 3*) or Mg^{2+} (*lane 2*), although both cations stimulate the binding when added together to the assay medium (*lane 5*).

In the absence of Ca^{2+} and Mg^{2+}, no labeling of proteins was detected (Fig. 9, *lane 4*), and a significant reduction of labeling was observed when native membranes (*lane 1*) were utilized instead of EDTA-washed membranes. It appears that incorporation of exogenous CaM occurs when most of the receptors are depleted of endogenous CaM, in agreement with the results depicted in Fig. 2.

Lateral cross-linking between membrane components appears not to be significant since the Coomassie blue staining pattern was not altered by the cross-linker concentrations used. However, the 102-kDa radioactive product was not detected by photoaffinity labeling (Fig. 8*B*), so that it is not ruled out that this product is a lateral cross-linking derivative obtained under certain conditions (presence of Mg^{2+}) of chemical cross-linking (Fig. 9, *lanes 2* and *5*).

DISCUSSION

The study shows that CaM interacts with skeletal SR in a cation-dependent manner. Ca^{2+} stimulates significantly the binding of CaM to the membranes, but Mg^{2+} has a more potent stimulatory effect which is distinct from that of Ca^{2+} (Fig. 1). The cation-dependent binding appears to be specific for CaM since it is competitively inhibited by trifluoperazine or unlabeled CaM (Figs. 4 and 5).

In the presence of optimal Ca^{2+} and Mg^{2+} concentrations, CaM specifically saturates SR membranes with a maximal binding capacity of about 5 pmol/mg of protein (Fig. 3). Under these conditions, one class of binding sites was observed which binds CaM with high affinity ($K_D \sim 52$ nM). On the other hand, when an optimal CaM concentration is maintained and the concentration of Ca^{2+} varies in the reaction medium, CaM binding is expressed in two saturable steps with $K_{m(Ca)}$ values of about 1.6×10^{-7} and 3×10^{-5} M, respectively (Fig. 6). It appears therefore that SR contains two types of CaM receptors with different sensitivity to the presence of Ca^{2+}. These observations agree with the idea that different conformations of CaM arise when Ca^{2+} is sequentially bound to its Ca^{2+}-binding sites, resulting in the activation of different CaM receptors (33).

The CaM-binding sites having a half-saturation of 1.6×10^{-7} M are probably associated with the adenylate cyclase which was recently found in SR by Nakagawa and Willner (34). The other type of CaM-binding sites with a $K_{m(Ca)}$ value of about 3×10^{-5} M corresponds probably to proteins whose identity is discussed below.

In contrast to Ca^{2+}, the sensitivity of the CaM binding process to Mg^{2+} is expressed in one step with a $K_{m(Mg)}$ value of about 2×10^{-3} M, which indicates that one type of Mg^{2+}-dependent CaM receptors exists in SR (Fig. 7). Although the Mg^{2+} effect is more potent than that of Ca^{2+}, the Mg^{2+} concentrations required for CaM binding (millimolar range) are higher than those of Ca^{2+} (micromolar range). This is in good agreement with the physiological concentrations of these cations within the cell: Mg^{2+} concentration is in the order of 10^{-3} M, whereas that of Ca^{2+} is 10^{-7} M in the resting state and about 10^{-5} M upon stimulation (35).

Ca^{2+} and Mg^{2+} have been described as CaM-binding stimulators in membranes of erythrocytes (36, 37), cardiac sarcolemma (38), synaptic membranes (39), and lens plasma membranes (40). However, only in SR do both cations appear to stimulate CaM binding by distinct mechanisms since additive effects can be observed when Ca^{2+} and Mg^{2+} are simultaneously added to the reaction medium. Probably, CaM exhibits Mg^{2+}-dependent conformers which are recognized by some CaM-binding proteins of the SR membranes. Indeed, Milos *et al.* (41) reported that CaM contains four Mg^{2+}-binding sites which are different from the Ca^{2+}-binding sites and that, at high concentrations of both cations, a CaM-Ca_4-Mg_4 species is formed. All Mg^{2+}-specific sites have the same affinity (41), which probably justifies the one type of Mg^{2+}-dependent CaM receptors observed in SR (Fig. 7). On the other hand, Tsai *et al.* (42) suggested that sites I and II of CaM are Mg^{2+}/Ca^{2+} sites, whereas sites III and IV are Ca^{2+} sites with regulatory properties. Considering the Mg^{2+} compartmentation in the cells (43), it is possible that Mg^{2+}, like Ca^{2+}, functions as a physiological regulator whose effect is CaM-mediated. In fact, Mg^{2+} has been observed as a coupling factor between Ca^{2+} transport and the ATP hydrolyzed by SR (44, 45), but it is not known whether CaM-binding proteins are involved in this process.

The components of SR which bind CaM in a cation-dependent manner were identified by autoradiography in sodium dodecyl sulfate gels (Figs. 8 and 9). Six ^{125}I-containing products which correspond to complexes between ^{125}I-CaM and SR proteins of about 148, 125, 60, 41, 33, and 23 kDa were detected either by photoaffinity cross-linking or by chemical cross-linking in the presence of Ca^{2+} and Mg^{2+}.

The proteins of about 148 and 125 kDa may correspond to the α and β subunits of the phosphorylase kinase which has been reported as an intrinsic component of SR (46), whereas the 85-kDa protein, detected by chemical cross-linking, and the proteins of about 60, 33, and 23 kDa have molecular masses similar to those previously described as components of the Ca^{2+} release channel (12) or of the CaM-dependent phosphorylating systems of skeletal SR (11, 14). If these proteins are CaM receptors, it appears that they accept P_i by a CaM-dependent autophosphorylation process. Indeed, the kinase enzyme that phosphorylates these substrates was never identified in SR. The 41,000-kDa CaM receptor may be a kinase protein since binding of 8-azido-[^{32}P]ATP to a protein of similar molecular mass was demonstrated by Campbell and MacLennan (47). However, no experimental evidence for the enzyme activity of this protein was obtained.

The 60-kDa protein which binds CaM in a Ca^{2+}- or Mg^{2+}-dependent way probably represents the 60-kDa component of the Ca^{2+} release channel. CaM-dependent phosphorylation of this component was found to inhibit Ca^{2+} release from SR vesicles (12). However, results of Meissner (18) indicate that ATP is not required and that inhibition of Ca^{2+} release is due to a direct interaction of CaM with the channel.

Another radiolabeled band containing high molecular weight proteins (>200,000) was detected in this work in agreement with the observations of Seiler *et al.* (22). However, these authors failed to observe most of the CaM-binding proteins reported here probably because endogenous CaM was not sufficiently removed under their experimental conditions. All CaM-binding proteins discussed here have molecular weights similar to proteins which were demonstrated to be integral components of the longitudinal tubules and of the terminal cisternae of SR (48). Therefore, it is unlikely that they are contaminants of the SR preparation used in this work.

The results reported here indicate that SR contains several types of cation-dependent CaM receptors and that CaM binding to them is determined by the effects of Ca^{2+} and Mg^{2+}. Experiments are currently in progress to clarify the function and exact identity of the CaM-binding proteins in skeletal SR membranes.

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