Calmodulin Interaction with the Skeletal Muscle Sarcoplasmic Reticulum Calcium Channel Protein†

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ABSTRACT: Studies were initiated to define the equilibria of calmodulin binding to the skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+}-release channel protein in native SR vesicles. Calmodulin affinity-labeling experiments indicated that the major calmodulin receptor in heavy SR preparations was a protein of $M_r > 450\,000$, corresponding to the Ca^{2+}-release channel protein. [^{3}H]Ryanodine-binding assays indicated 10.6 ± 0.9 pmol of high-affinity ryanodine binding per milligram of SR protein. Wheat germ calmodulin was derivatized with rhodamine-*x*-maleimide. The affinity and binding capacity of the channel protein in SR vesicles for the derivatized calmodulin (Rh-CaM) were determined by fluorescence anisotropy in the presence of (1) 1 mM EGTA, (2) 0.1 mM $CaCl_2$, and (3) 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$. In the presence of EGTA, Rh-CaM bound to the channel protein with a K_d of 8.6 ± 0.8 nM and a B_{max} of 229 ± 7 pmol/mg, suggesting that calmodulin binds to the channel protein at [Ca^{2+}] comparable to that in resting muscle. In the presence of 0.1 mM $CaCl_2$, the binding equilibrium shifted to a two-site ligand-binding model; the high-affinity class of sites had a B_{max1} of 54 ± 7 pmol/mg and a K_{d1} of 4.3 ± 1.1 nM, while the lower affinity class of sites had a B_{max2} of 166 ± 28 pmol/mg and a K_{d2} of 239 ± 102 nM. In the presence of both Ca^{2+} and Mg^{2+}, there was a further change in the Rh-CaM/channel protein interaction. B_{max1} was 10.0 ± 0.8 pmol/mg, and K_{d1} was 0.10 ± 0.03 nM; B_{max2} was 70 ± 2 pmol/mg, and K_{d2} was 17 ± 1 nM. These data are consistent with the hypothesis that there are multiple calmodulin-binding sites on each channel protein subunit with the affinities of these calmodulin-binding sites depending on the concentrations of Ca^{2+} and Mg^{2+}. Thus, the binding of calmodulin to the SR Ca^{2+} channel is regulated by modulators of the Ca^{2+} channel activity itself, and this novel regulation is likely to be important in the mechanism of excitation–contraction.

Contraction and relaxation of skeletal muscle are governed by changes in myoplasmic [Ca^{2+}]. Elevation of myoplasmic [Ca^{2+}], which triggers contraction, occurs when an action potential induces Ca^{2+} release from the sarcoplasmic reticulum (SR)[1] via a Ca^{2+}-release channel (Martonosi, 1984; Inesi, 1985). Upon cessation of the action potential, Ca^{2+} release is inhibited, the SR Ca^{2+}-pump protein reduces myoplasmic [Ca^{2+}] by pumping it back into the SR, and the muscle relaxes. Although the Ca^{2+} uptake process has been described in considerable detail, the molecular mechanisms involved in Ca^{2+} release are, by comparison, poorly understood.

Regulation of Ca^{2+}-release channel activity has been investigated by following Ca^{2+} efflux from isolated SR membrane vesicles (Nagasaki & Kasai, 1983; Ikemoto et al., 1985; Meissner et al., 1986; Meissner, 1986) as well as by single-channel recordings of the activity of the Ca^{2+}-release channel protein incorporated from SR vesicles into planar lipid bilayers (Smith et al., 1985, 1986a,b). Results of these experiments indicated the presence of a Ca^{2+} channel protein in SR whose activity was stimulated by Ca^{2+}, caffeine, and adenine nucleotides and nanomolar concentrations of the plant alkaloid ryanodine; channel activity was inhibited by Mg^{2+}, calmodulin (CaM), and ruthenium red and micromolar concentrations of ryanodine.

Recently several groups have isolated a homotetrameric protein of subunit $M_r > 450\,000$ which bound ryanodine and, when incorporated into planar lipid bilayers, exhibited Ca^{2+} channel activity nearly identical to that of intact SR vesicles and native SR Ca^{2+} channels (Inui et al., 1987; Lai et al., 1988; Imagawa et al., 1987). The purified ryanodine receptor/Ca^{2+} channel protein also displayed strikingly similar morphology to the junctional foot structure which spans the gap between the T-tubule and junctional SR (Inui et al., 1987; Lai et al., 1988). On the basis of this evidence, the ryanodine receptor protein was proposed to be synonymous with the Ca^{2+}-release channel and the junctional foot of SR vesicles.

Calmodulin is a ubiquitous Ca^{2+}-binding protein which regulates the activity of at least 30 known enzymes and other proteins in response to changes in cellular Ca^{2+} concentration (Klee & Vanaman, 1982; Means et al., 1991). Previous studies have shown that CaM inhibits the Ca^{2+}-release rate from SR vesicles by 2–3-fold in skeletal muscle (Meissner, 1986; Plank et al., 1988) and up to 6-fold in cardiac muscle (Meissner & Henderson, 1987). Subsequently, Smith et al. (1989), using the planar lipid bilayer–vesicle fusion technique, demonstrated that the inhibitory effect of CaM on both the skeletal and cardiac SR channel proteins resulted from reduction of the open state probability via direct binding of CaM to the channel protein.

These results have led to the hypothesis that the role of CaM in regulation of the SR Ca^{2+} channel activity is that of a partial feedback inhibitor of Ca^{2+} release (Meissner, 1986;

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[1] Abbreviations: SR, sarcoplasmic reticulum; CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Rh, rhodamine-*x*-maleimide; Bz, benzophenone-4-maleimide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; B_{max}, maximal number of binding sites; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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Smith et al., 1989). However, in the absence of direct binding data on the interaction of CaM with the channel protein, it is difficult to assess the physiological role of CaM in regulation of Ca^{2+}-release activity. These studies were initiated to define conditions under which CaM binds to the channel protein and, thus, would be capable of regulating its activity. Our results suggest that in skeletal heavy SR, the most abundant receptor for CaM is the Ca^{2+} channel protein. CaM binds to the channel protein with high affinity, even in the presence of EGTA. In the physiological range of KCl concentrations, binding of CaM is enhanced in the presence of 0.1 mM $CaCl_2$, and further enhanced by inclusion of 1 mM $MgCl_2$. Correlation of ryanodine-binding data with CaM-binding data suggests that there are multiple CaM-binding sites on each channel protein subunit and that the affinities of these binding sites for CaM change in response to metal ion concentrations.

EXPERIMENTAL PROCEDURES

Materials. Benzophenone-4-maleimide and rhodamine-*x*-maleimide were purchased from Molecular Probes (Junction City, OR). $Na^{125}I$ and [^{3}H]ryanodine were obtained from DuPont–NEN (Boston, MA). Ryanodine was purchased from Calbiochem (La Jolla, CA). Wheat germ was a generous gift from International Multifoods (Minneapolis, MN).

Preparation of Calmodulin and Its Derivatives. CaM was purified from wheat germ using the procedure described previously (Strasburg et al., 1988). For cross-linking experiments, CaM was iodinated with ^{125}I at the sole tyrosine residue (Tyr-139), followed by site-specific modification at Cys-27 with the photoactivatable cross-linker benzophenone-4-maleimide (Strasburg et al., 1988). For fluorescence experiments, purified CaM was derivatized at Cys-27 with rhodamine-*x*-maleimide as described for I-EDANS (Strasburg et al., 1988).

Calmodulin Cross-Linking. Affinity labeling of CaM-binding proteins in SR vesicles was performed by incubating in darkness 0.1 μM [^{125}I]-Bz-CaM with 100 μg of SR vesicles in 100 μL of 20 mM Hepes, pH 7.5, and 0.2 M NaCl. $CaCl_2$, $MgCl_2$, and EGTA were included as indicated in the figure legends. The mixtures were placed in plastic microcentrifuge tubes on ice, the tubes were covered with a plate of glass, and the samples were illuminated for 20 min in a Stratalinker 1800 photoreactor (Stratagene Corp., La Jolla, CA) equipped with lamps of λ_{max} = 254 nm. After photolysis of the mixture, the samples were centrifuged in a Beckman TL-100 centrifuge at 100000g_{max} for 20 min. The membrane pellets were resuspended in water, SDS was added to produce a final concentration of 1%, and the samples were subjected to polyacrylamide gel electrophoresis using 5–20% linear gradient gels (Laemmli, 1970). The gels were dried and placed with Kodak Omat XAR-5 X-ray film in autoradiography cassettes equipped with Dupont Lightning Plus intensifying screens.

Preparation of Sarcoplasmic Reticulum Vesicles. Skeletal muscle heavy SR vesicles were isolated from longissimus muscle of pigs obtained from the Michigan State University swine farm or from the Yorkshire swine herd at the University of Minnesota using the procedure of Mickelson et al. (1986) with the modifications that 1 mM EGTA, 0.1 mM PMSF, 1 μg/mL aprotinin, and 1 μg/mL leupeptin were included in the homogenization buffer. Light SR vesicles were prepared from rabbit longissimus muscles according to the procedure of Fernandez et al. (1980).

Fluorescence Anisotropy Measurements. Rhodamine maleimide-labeled CaM (Rh-CaM) binding to the channel protein in SR vesicles was monitored by fluorescence anisotropy measurements using an SLM 4800 spectrofluorometer modified with data acquisition hardware and operating system from On-Line Instrument Systems (Bogart, GA).

Samples were held in a thermostated cell block maintained at 22 °C. The excitation wavelength of Rh-CaM was 580 nm, monochromator slits were set at 8 nm, and emitted light was isolated using Schott RG-610 filters. During titrations, samples were allowed to equilibrate for 5 min after each addition of Rh-CaM or SR. All samples included 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.1 mM PMSF to inhibit proteolysis during the experiment.

All fluorescence measurements were performed using semimicro, quartz fluorescence cuvettes (4 mm × 10 mm). Prior to fluorescence experiments, the cells were rinsed with 1 mg/mL bovine serum albumin to minimize Rh-CaM adsorption to the walls of the cuvette. Following this treatment, the measured anisotropy value for Rh-CaM was independent of concentration over the range of 1 nM to 1000 nM, indicating that there was negligible Rh-CaM adsorption to the cuvettes.

The anisotropy of a ligand (Rh-CaM) is directly proportional to the fraction of ligand bound to the receptor (Ca^{2+} channel protein). Thus, if A_f is the anisotropy of free Rh-CaM and A_b is the anisotropy of the fully bound ligand, then the fraction bound, f_b, is determined from

$$f_b = \frac{A_m - A_f}{A_m(1 - q) + qA_b - A_f} \quad (1)$$

where A_m is the measured anisotropy for a given ligand concentration, and q, the change in quantum yield, is the ratio of the fluorescent intensity of the bound species over that of the free species. If the change in quantum yield is negligible upon binding of ligand, then eq 1 reduces to

$$f_b = \frac{A_m - A_f}{A_b - A_f} \quad (2)$$

The fraction of ligand bound and the concentrations of bound and free Rh-CaM are readily calculated. Values of K_d and B_{max} were calculated using the computer program Enzfitter, a nonlinear regression analysis program of R. J. Leatherbarrow (Biosoft, Cambridge, U.K.). Data were fit to a one-ligand- or two-ligand-binding model to obtain the best fit.

The anisotropy of unbound Rh-CaM, A_f, was measured in the absence of SR. The anisotropy of the fully bound species, A_b, was obtained by titration of Rh-CaM with SR vesicles, followed by curve-fitting using the Enzfitter computer program for a single class of ligand-binding sites. Corrections for light scattering and background fluorescence were made by application of the equation:

$$A = f_1A_1 + f_2A_2 \quad (3)$$

where A, A_1, and A_2 are the anisotropies of the sample, the blank, and the corrected sample, respectively. The fractional contributions, f_1 and f_2, of these species were calculated from the intensities measured with the excitation monochromator in the vertical position and the emission monochromator at 55°. The corrected sample anisotropy, therefore, is that value in the absence of background interference.

Calmodulin Content of Heavy SR Vesicles. Heavy SR vesicles were suspended to a concentration of 1 mg/mL in 20 mM imidazole buffer, pH 7.4. The suspension was incubated for 5 min in a boiling water bath and centrifuged for 15 min at 100000g_{max} in a Beckman TL-100 centrifuge. Aliquots of the CaM-containing supernatant were used in the erythrocyte membrane ghost CaATPase assay described below.

Preparation of porcine erythrocyte membrane ghosts and assays for CaM-stimulatable activity of the CaATPase of the

erythrocyte ghosts were based on the procedures described by Thatte et al. (1987). Inorganic phosphate was determined by the method of Rockstein and Herron (1951). A standard curve was prepared for CaATPase activity as a function of wheat germ CaM concentration; the CaM content of heavy SR vesicles was determined from the standard curve using aliquots of the boiled SR supernatants. CaM-stimulatable CaATPase activity of the boiled supernatants was defined as the ATPase activity in the presence of 0.1 mM $CaCl_2$ minus that of an equal aliquot in the presence of 1 mM EGTA.

Biochemical Assays. SR protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. Ryanodine-binding activity of SR vesicles was determined according to the method of Mickelson et al. (1988). Ca^{2+} titration of CaM/channel protein complexes was performed using an EGTA/NTA buffer. Free Ca^{2+} concentrations were calculated using the computer program of Perrin and Sayce (1967).

RESULTS

Calmodulin Content of Heavy SR Vesicles. The CaM content of porcine heavy SR vesicles was determined by heating SR samples to release CaM, centrifugation to remove insoluble material, and measuring CaM activity in an erythrocyte membrane ghost CaATPase assay. The CaM content of our preparations of heavy SR ranged from 15 to 33 pmol/mg. Prior extractions of SR vesicles with EGTA did not significantly affect these results, indicating that the endogenous CaM was tightly bound and likely nonexchangeable.

[^3H]Ryanodine Binding to SR Vesicles. The binding activity of [^{3}H]ryanodine to heavy SR vesicles, determined by Scatchard plot analysis, shows a B_{max} value of 10.6 ± 0.9 pmol/mg for our preparations. These results were similar to those obtained by Mickelson et al. (1988). The ryanodine-binding activity of the rabbit skeletal muscle light SR vesicles was 0.1 pmol/mg.

Identification of Calmodulin-Binding Proteins in SR Vesicles. Purified heavy SR vesicles from porcine skeletal muscle and purified light SR from rabbit skeletal muscle were incubated with the affinity-labeling derivative [^{125}I]-Bz-CaM to identify the receptor proteins for CaM. The autoradiogram (Figure 1) of the gel electrophoretogram indicates that the major complex formed in the heavy SR fraction was a doublet of $M_r > 450\,000$ which corresponds to CaM plus the channel protein subunit (Seiler et al., 1984). This complex was obtained in the presence of EGTA or Ca^{2+} at each Mg^{2+} concentration examined and suggests that CaM could bind to the channel protein at [Ca^{2+}] in resting muscle. In contrast, at the same protein concentrations and under the same conditions, there was no apparent affinity-labeling of light SR (Figure 1B). In order to demonstrate that the binding of CaM to the Ca^{2+} channel protein was specific, affinity-labeling experiments were conducted in the presence of increasing concentrations of unlabeled CaM. The [^{125}I]-Bz-CaM was readily displaced from the channel protein by the unlabeled CaM (Figure 1C), with cross-linking eliminated at unlabeled CaM concentrations greater than 1 μM.

Titration of Rh-CaM with SR Vesicles. Since the affinity-labeling experiments indicate that the Ca^{2+} channel protein is the most abundant receptor for CaM in our heavy SR preparations, fluorescence anisotropy could be used to characterize the CaM interaction with the channel protein in native SR vesicles. Heavy SR vesicles were titrated into Rh-CaM under three different metal ion conditions: (1) +1 mM EGTA; (2) +0.1 mM $CaCl_2$; (3) +0.1 mM $CaCl_2$ and 1 mM $MgCl_2$.

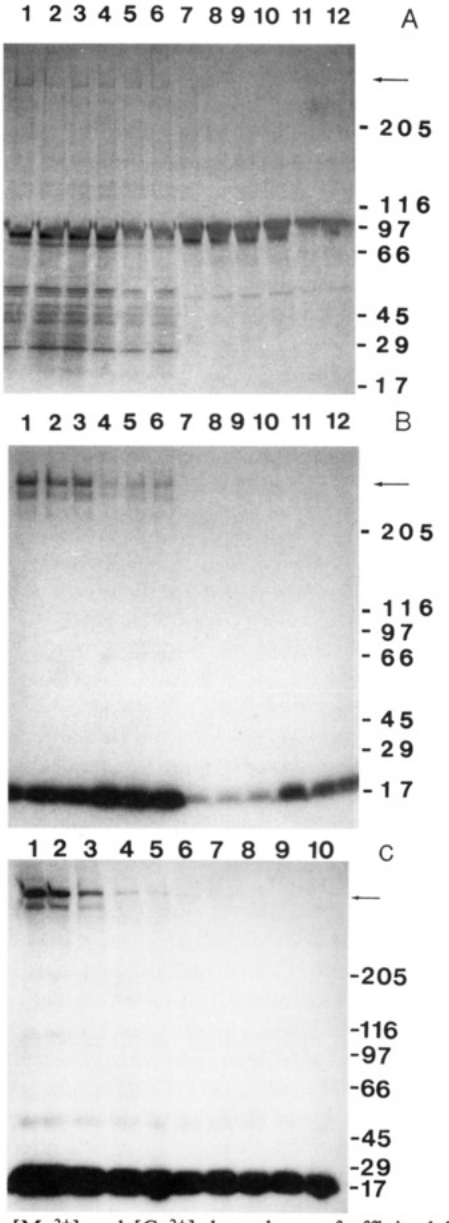

FIGURE 1: [Mg^{2+}] and [Ca^{2+}] dependence of affinity labeling of skeletal muscle heavy and light SR with [^{125}I]-Bz-CaM. [^{125}I]-Bz-CaM was incubated with skeletal muscle heavy SR vesicles (A and B, lanes 1–6) or light SR vesicles (A and B, lanes 7–12) in 0.2 M NaCl/20 mM Hepes buffer (pH 7.0) plus the following components: lanes 1, 7, 1 mM EGTA; lanes 2, 8, 1 mM EGTA, 1 mM $MgCl_2$; lanes 3, 9, 1 mM EGTA, 10 mM $MgCl_2$; lanes 4, 10, 0.1 mM $CaCl_2$; lanes 5, 11, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$; lanes 6, 12, 0.1 mM $CaCl_2$, 10 mM $MgCl_2$. (A) Coomassie blue stained gel. (B) Autoradiogram of dried gel. (C) Inhibition of affinity labeling of skeletal muscle heavy SR vesicles by [^{125}I]-Bz-CaM with unlabeled CaM. Skeletal muscle heavy SR vesicles (1 mg/mL) were incubated with 0.1 μM [^{125}I]-Bz-CaM in the presence of 0.2 M NaCl, 20 mM Hepes buffer, pH 7.5, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and varying amounts of unlabeled CaM. The mixtures were separated by electrophoresis on a 5–20% acrylamide gradient gel, and the cross-linked products were identified by autoradiography of the gel. Lanes 1–10 represent 0, 0.05, 0.1, 0.5, 1, 2, 4, 6, 8, and 10 μM unlabeled CaM, respectively. The arrow indicates the position of the channel protein subunit in the gel, and of the channel subunit/[^{125}I]-Bz-CaM complex in the autoradiograms.

In each case, titration of SR vesicles into Rh-CaM resulted in a large increase in fluorescence anisotropy attributable to the increased molecular mass of the Rh-CaM/Ca^{2+} channel protein complex (Figure 2). That the Rh-CaM was indeed binding to the channel protein is supported by the affinity-labeling experiments (Figure 1B) and the following control

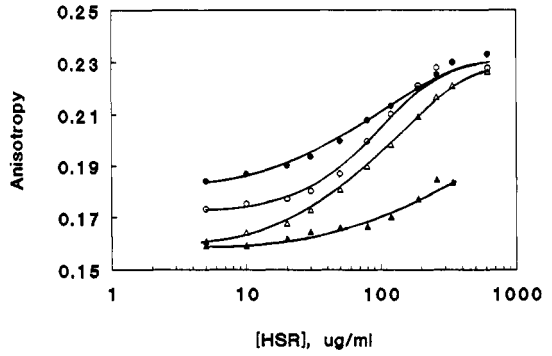

FIGURE 2: Titration of Rh-CaM with skeletal heavy and light SR vesicles under different divalent ion conditions. The sample medium contained 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, and 50 mM Pipes, pH 7.0, plus one of the following conditions: 1 mM EGTA (●); 0.1 mM $CaCl_2$ (○); 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (△, ▲) in a starting volume of 1 mL. Heavy SR (○, ●, △); light SR (▲). The Rh-CaM sample was titrated with SR vesicles in parallel with a buffer blank containing the same media minus Rh-CaM. Corrections were made for light scattering as described under Experimental Procedures.

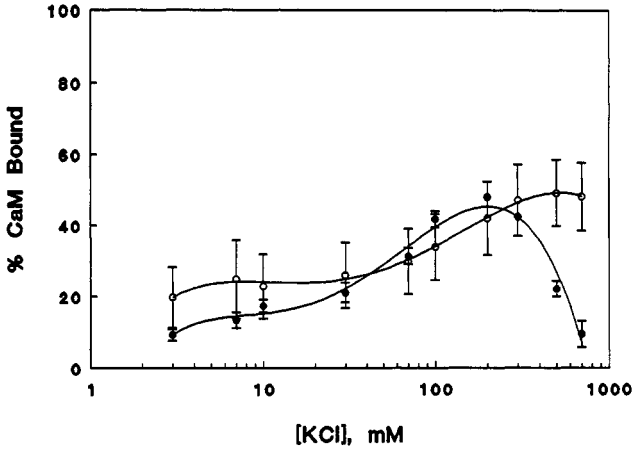

FIGURE 3: [KCl] dependence of the binding of Rh-CaM to SR vesicles under different divalent ion conditions. The sample buffer contained 86 μg of heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 50 mM Pipes, pH 7.0, and either 1 mM EGTA (●) or 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ (○) in a starting volume of 1 mL. Points represent the means ● SE of three preparations. Percent CaM bound was calculated from measured anisotropy as described under Experimental Procedures; 100% bound corresponds to 229 pmol/mg in the presence of EGTA and 80 pmol/mg in the presence of calcium plus magnesium.

experiments. The increase in anisotropy was reversed by addition of a large excess of unlabeled CaM (not shown). Titration of light SR vesicles into Rh-CaM resulted in a slight increase in fluorescence only at high SR concentrations (Figure 2). Furthermore, titration of Rh-CaM with SR vesicles, which were first treated with trypsin (1:50 w/w, 1 h at 37 °C), resulted in no change in anisotropy (not shown), indicating that there were negligible nonspecific Rh-CaM interactions with the membranes and that light-scattering corrections were valid.

Data from these titrations (Figure 2) were used to determine the anisotropy values for the free Rh-CaM species (A_f) and for the fully bound Rh-CaM/Ca^{2+} channel protein complex (A_b). Normally these values are obtained from the limits of the titration curves. However, values for A_b could be slightly underestimated in these experiments because of excessive light scattering at high concentrations of SR vesicles (>1 mg/mL). Instead, A_b values were calculated by extrapolation using the Enzfitter program applied for a single ligand-binding site on Rh-CaM. The A_b values obtained for each metal ion condition averaged 0.2435. The A_f values obtained from 10 nM samples of Rh-CaM, under the various metal ion conditions in the absence of added SR, were 0.1808 (+1 mM EGTA), 0.1728 (+0.1 mM $CaCl_2$), and 0.1585 (+0.1 mM $CaCl_2$, +1 mM $MgCl_2$). There was no significant change in fluorescence intensity upon binding of Rh-CaM to the channel protein (q = 1.0); therefore, the fraction of Rh-CaM bound was calculated using eq 2.

Ionic Strength Dependence of the Binding of Rh-CaM to SR Vesicles. Our initial experiments (not shown) were conducted in the absence of added KCl and showed that in the presence of EGTA there was no change in Rh-CaM anisotropy upon titration with SR vesicles. The results thus suggested that in the absence of KCl there was no binding of Rh-CaM to the channel protein in the presence of <50 nM Ca^{2+}. However, in the presence of KCl, there was an increase in fluorescence anisotropy observed upon titration of SR into Rh-CaM in the presence of EGTA (Figure 2). To determine whether binding of Rh-CaM to the skeletal SR Ca^{2+} channel protein could be dependent upon ionic strength, the anisotropy for fixed concentrations of Rh-CaM and SR vesicles was measured as a function of KCl concentration. The chosen concentrations of SR (86 μg) and Rh-CaM (10 nM) correspond to the approximate midpoints of the titration curves in Figure 2. As the salt concentration was varied, either an enhancement or an inhibition of binding would be observed by an increase or decrease in anisotropy, respectively. The KCl titration data (Figure 3) clearly show that in the presence of EGTA, the binding of Rh-CaM to the channel protein was highly ionic strength dependent, increasing from <10% Rh-CaM bound at 3 mM KCl to 45% bound at 0.2 M KCl. At [KCl] >0.3 M, the anisotropy rapidly declined, indicating decreased binding of Rh-CaM to the Ca^{2+} channel protein. In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, titration of KCl into the SR/Rh-CaM mixture also indicated a significantly higher affinity of Rh-CaM for the Ca^{2+} channel protein, with a maximum at about 0.3 M KCl. However, in contrast to the EGTA conditions, binding of Rh-CaM to the channel protein did not significantly decrease at higher KCl concentrations.

[Ca^{2+}] Dependence of the Binding of Rh-CaM to SR Vesicles. Ca^{2+} is required for the binding of CaM to most of its known receptors (Klee et al., 1982), although in a few cases the affinity of CaM for its receptor decreases with elevated [Ca^{2+}] (Cimler et al., 1985; Masure et al., 1986). Therefore, to define optimal [Ca^{2+}] for CaM/channel protein interaction, the [Ca^{2+}] dependence of the binding of Rh-CaM was determined. The optimal [Ca^{2+}] for Rh-CaM/channel protein interaction was dependent on the $MgCl_2$ concentration (Figure 4). In the absence of Mg^{2+}, the optimal [Ca^{2+}] for enhanced binding of Rh-CaM to the channel protein was approximately 1 μM, whereas in the presence of 1 mM Mg^{2+}, the optimum [Ca^{2+}] for Rh-CaM binding to the channel protein was approximately 50 μM (Figure 4).

[Mg^{2+}] Dependence of the Binding of Rh-CaM to SR Vesicles. Previous results (Meissner et al., 1986) indicated that Mg^{2+} is an antagonist of the Ca^{2+}-release activity in heavy SR vesicles. To determine whether Mg^{2+} might alter the interaction of CaM with the Ca^{2+} channel protein, the anisotropy of Rh-CaM was determined as a function of [Mg^{2+}] in the presence or absence of 0.1 mM Ca^{2+}. Binding of Rh-CaM to the channel protein was slightly enhanced as the [Mg^{2+}] was increased to 0.5 mM (+EGTA) or 1 mM (+Ca^{2+}) as indicated by the increase in fluorescence anisotropy. At [Mg^{2+}] above 1 mM (+EGTA) or above 7 mM (+Ca^{2+}), binding was significantly inhibited (Figure 5).

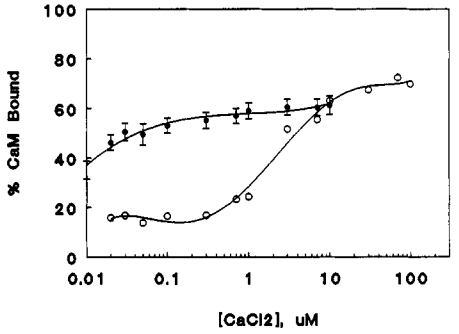

FIGURE 4: Titration of Rh-CaM/SR vesicles with $CaCl_2$. The sample medium contained 86 μg of heavy SR vesicles, 10 nM CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM Pipes, pH 7.0, and either 1 mM $MgCl_2$ (○) or no $MgCl_2$ (●) in a starting volume of 1 mL. Points represent the means ± SE of three preparations. Percent CaM bound was calculated from measured anisotropy as described under Experimental Procedures; 100% CaM bound corresponds to 229 pmol/mg in the absence of magnesium and 80 pmol/mg in the presence of magnesium.

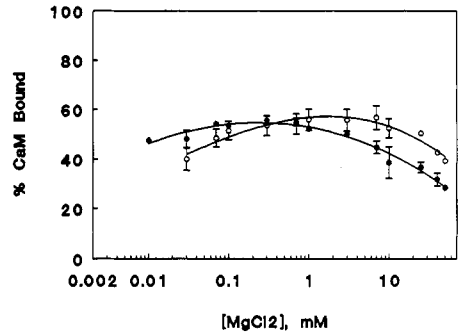

FIGURE 5: Titration of Rh-CaM/SR vesicles with $MgCl_2$. The sample medium contained 86 μg of heavy SR vesicles, 10 nM Rh-CaM, 0.3 M sucrose, 0.3 M KCl, 50 mM Pipes, pH 7.0, and 0.1 mM $CaCl_2$ (○) or 1 mM EGTA (●) in a starting volume of 1 mL. Points represent the means ± SE of three preparations. Percent CaM bound was calculated from measured anisotropy as described under Experimental Procedures; 100% bound corresponds to 80 pmol/mg in the presence of calcium and 229 pmol/mg in the presence of EGTA.

Rh-CaM/Ca^{2+} Channel Protein Binding Equilibrium. Having established optimal salt and divalent metal ion concentrations, Rh-CaM was titrated into fixed concentrations of SR vesicles to determine the binding capacity and the affinity of the skeletal SR Ca^{2+} channel in SR vesicles for Rh-CaM under the following conditions: (1) 1 mM EGTA; (2) 0.1 mM $CaCl_2$; (3) 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$. A_f and A_b values were used to calculate the fraction of Rh-CaM bound to the channel protein in SR vesicles for each point in the titration.

Results of titrations conducted in the presence of EGTA are indicative of a single class of binding sites on the SR Ca^{2+} channel protein for CaM (Figure 6 and Table 1). Scatchard analysis of the data yields a dissociation constant, K_d, of 8.6 ± 0.8 nM and a binding capacity, B_{max}, of 229 ± 7 pmol/mg of SR protein. In the presence of 0.1 mM $CaCl_2$, the titration data are consistent with two classes of ligand-binding sites on the channel protein for CaM (Figure 7 and Table 1). The high-affinity class of sites has K_{d1} = 4.3 ± 1.1 nM and B_{max1} = 54 ± 7 pmol/mg; the results of low-affinity-binding class site show K_{d2} = 239 ± 102 and B_{max2} = 166 ± 28 pmol/mg. In the presence of 0.1 mM Ca^{2+} plus 1 mM Mg^{2+}, there is a dramatic shift in the binding capacity of the high-affinity class of sites (Figure 8 and Table 1). The high-affinity-binding site has a B_{max1} = 10.0 ± 0.8 pmol/mg and K_{d1} = 0.10 ± 0.03 nM; the lower affinity class of sites has a K_{d2} of 17 ± 1 nM and a B_{max2} = 70 ± 2 pmol/mg.

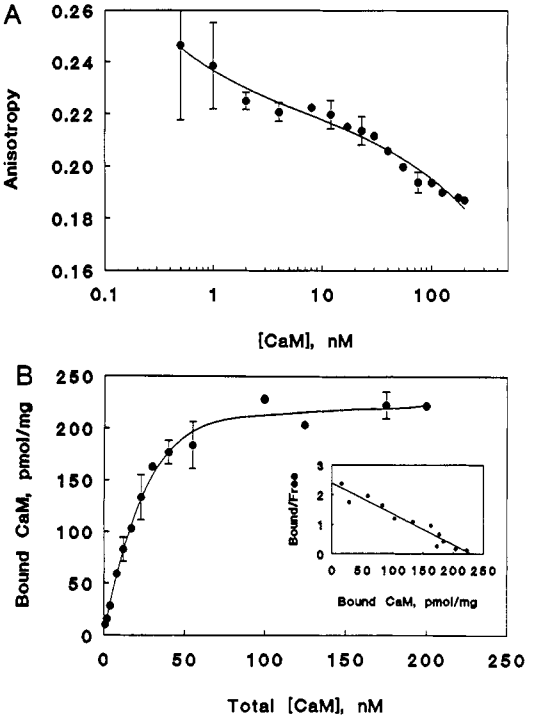

FIGURE 6: Titration of skeletal heavy SR vesicles with Rh-CaM in the presence of EGTA. (A) Anisotropy plot of titrations of heavy SR with Rh-CaM. (B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of Rh-CaM binding to SR vesicles. The sample medium contained 90 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM Pipes, pH 7.0, and 1 mM EGTA in a starting volume of 1 mL. Points represent the means ± SE of three preparations. CaM bound was calculated from the measured anisotropy as described under Experimental Procedures.

Table 1: Equilibrium Constants for Rh-CaM Interaction with the Ca^{2+} Channel Protein in SR Vesicles[a]

	B_{max1} (pmol/mg)	K_{d1} (nM)	B_{max2} (pmol/mg)	K_{d2} (nM)
+1 mM EGTA	229 ± 7	8.6 ± 0.8		
+0.1 mM $CaCl_2$	54 ± 7	4.3 ± 1.1	166 ± 28	239 ± 102
+0.1 mM $CaCl_2$, +1 mM $MgCl_2$	10.0 ± 0.8	0.1 ± 0.03	70 ± 2	17 ± 1

[a] Data were obtained from titrations of SR vesicles with Rh-CaM in the presence of 0.3 M KCl, 50 mM Pipes, pH 7.0, and divalent ion conditions as listed below. Data are ±SE of the means of three preparations each.

DISCUSSION

It has been previously shown that CaM partially inhibits Ca^{2+} release from SR vesicles (Meissner, 1986; Plank et al., 1988) and lowers the open-state probability of the channel protein in planar lipid bilayers (Smith et al., 1989). The present studies were conducted to characterize the interaction of CaM with the Ca^{2+} channel protein in heavy SR vesicles by defining conditions in which CaM may bind to and thus potentially regulate channel protein activity.

The porcine heavy SR vesicles used in this study contained a small but significant amount of CaM. The fact that subsequent EGTA washes of the vesicles prior to the endogenous CaM assay did not significantly reduce CaM levels suggests that the endogenous CaM is nonexchangeable and tightly bound to proteins such as phosphorylase kinase (Eibschutz et al., 1984). Our results for endogenous CaM are slightly higher than those obtained by Meissner (1986), who obtained 6–12 pmol/mg of protein for rabbit skeletal muscle heavy SR vesicles. The differences may result from

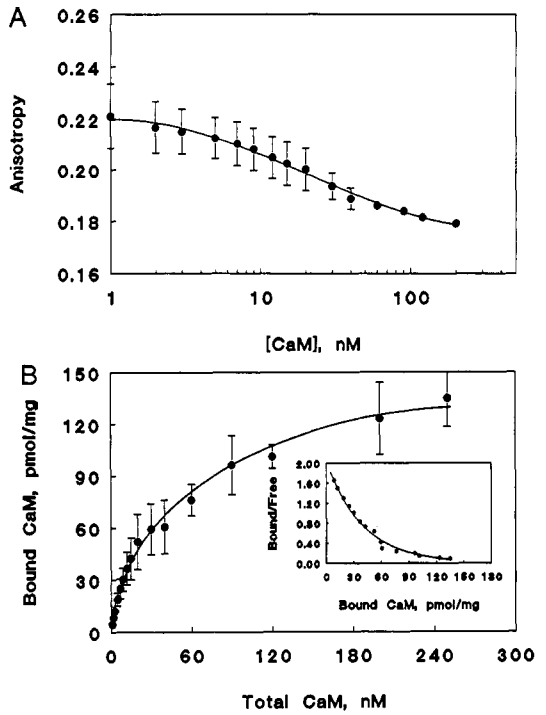

FIGURE 7: Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$. (A) Anisotropy plot of titrations of heavy SR with Rh-CaM. (B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot of binding of Rh-CaM to SR vesicles. The sample medium contained 150 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM Pipes, pH 7.0, and 0.1 mM $CaCl_2$ in a starting volume of 1 mL. Points represent the means ± SE of three preparations. CaM bound was calculated from measured anisotropy as described under Experimental Procedures.

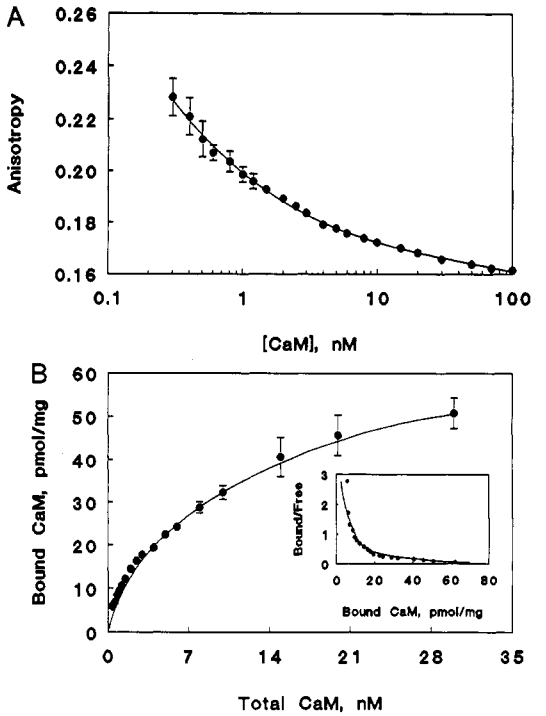

FIGURE 8: Titration of skeletal heavy SR with Rh-CaM in the presence of $CaCl_2$ plus $MgCl_2$. (A) Anisotropy plot of titration of heavy SR with Rh-CaM. (B) Rh-CaM/SR saturation binding curve. The inset is a Scatchard plot analysis of the binding of Rh-CaM to SR vesicles. The sample medium contained 50 μg of heavy SR, 0.3 M sucrose, 0.3 M KCl, 50 mM Pipes, pH 7.0, and 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$ in a starting volume of 1 mL. Points represent the means ± SE of three preparations. CaM bound was calculated from the measured anisotropy as described under Experimental Procedures.

species differences and in methods for determination of endogenous CaM abundance.

Affinity-labeling experiments were conducted to identify the CaM-binding proteins in our SR preparations. Wheat germ CaM, iodinated at Tyr-139, was derivatized at Cys-27 (located in the N-domain of CaM) with the photoaffinity label benzophenone-4-maleimide (Strasburg et al., 1988). This probe is reactive with any methylene groups of amino acid residues in the proximity of the label, and, thus, proteins which bind within 1.0 nm of the labeled Cys on CaM are readily cross-linked and identified by autoradiography. Our results indicated that the major receptor for CaM in purified heavy SR preparations was the Ca^{2+} channel protein and that the binding was specific (Figure 1). Light SR vesicles from rabbit skeletal muscle were devoid of Ca^{2+} channel protein as indicated by the low ryanodine-binding activity, the absence of a stained band in the gel, and the absence of affinity-labeled channel product in the autoradiogram (Figure 1). The amount of cross-linked product in heavy SR was somewhat greater in the presence of 1 mM EGTA than in the presence of 0.1 mM $CaCl_2$. However, cross-linking should not be regarded as a quantitative method. Differences in the amount of cross-linked product could reflect actual differences in the amount of CaM bound, but the fluorescence data (Table 1) indicated that this was not always the case. Transient, low-affinity complexes are quickly locked in a covalent cross-link upon excitation by light. Another explanation for the decreased yield in the presence of Ca^{2+} is decreased efficiency of cross-linking owing to a Ca^{2+}-dependent conformational change in the vicinity of the cross-linker on CaM or a Ca^{2+}-dependent conformational change in the channel protein.

Seiler et al. (1984) first observed that the primary CaM receptor in heavy SR was a protein doublet corresponding to the channel protein (Lai et al., 1988). Their experiments were done using mammalian CaM derivatized with the photoaffinity label methyl 4-azidobenzimidate which labels up to four different lysine residues on CaM (Klevit & Vanaman, 1984).

In contrast to these studies, Vale (1988) observed one major CaM receptor (60 kDa) and six minor CaM receptors (molecular mass >200, 148, 125, 41, 33, and 23 kDa) in SR membranes of rabbit skeletal muscle. The discrepancy may be largely attributable to the fact that Vale used a preparation of SR which was not fractionated on sucrose gradients. That preparation, therefore, would likely have comprised a more diverse membrane fraction which contained many more CaM-binding proteins than our preparations or those of Seiler et al. (1984). Furthermore, it is possible that proteolysis of the channel protein yielded fragments in Vale's preparations which bound CaM. Our preparation employed protease inhibitors to minimize this possibility.

Since there was one major receptor for CaM present in our heavy SR preparations, fluorescence spectroscopic techniques could be used to obtain quantitative data on the interaction of CaM with the channel in SR vesicles. Wheat germ CaM was chosen for derivatization because it possesses a single sulfhydryl residue for chemical modification (Toda et al., 1985). Rhodamine-*x*-maleimide was chosen for labeling of wheat germ CaM because the rhodamine–CaM adduct retains biological activity, it has a high quantum yield, and its emission maximum is in the red portion of the spectrum, thus providing a suitably strong signal in the low nanomolar concentration range of labeled CaM such that the light-scattering contribution to the total signal from the membrane vesicles is minimized (Mills et al., 1988; Strasburg et al., 1988).

CaM is known to regulate more than 30 different proteins and enzymes in a Ca^{2+}-dependent manner; i.e., Ca^{2+} first binds to CaM, inducing a conformational change in CaM which results in binding of the Ca_4CaM complex to the target protein and modulation of activity [see Klee and Vanaman (1982) for a review]. An exception to this mechanism is the interaction of CaM with the neural-specific protein P-57 or neuromodulin, in which binding of CaM to this receptor protein is enhanced in the presence of EGTA and reduced in the presence of Ca^{2+} (Andreasen et al., 1983; Masure et al., 1986; Cimler et al., 1985). A novel aspect emerging from our fluorescence studies on CaM binding to the channel protein is that, in the concentration range of 0.1–0.3 M KCl (i.e., in the range of physiological ionic strength), Rh-CaM bound with high affinity to the channel protein in the presence of EGTA. KCl had a dramatic effect on the affinity of the channel protein for CaM. At low ionic strength in the presence of EGTA, binding of CaM to the channel protein was minimal (Figure 3). Titration of a mixture of Rh-CaM plus SR vesicles with KCl resulted in strong enhancement of the affinity of the channel protein for Rh-CaM, possibly owing to an ionic strength-dependent conformational change in the channel protein. Some enhancement of binding was also noted when the KCl titration was conducted in the presence of 0.1 mM $CaCl_2$ plus 1 mM $MgCl_2$, although a greater fraction of Rh-CaM was bound initially and there was little change in affinity at high [KCl]. These results are complemented by the affinity-labeling experiments (Figure 1B) which show CaM cross-linking to the channel protein in the presence of EGTA. Together, these data suggest that CaM binds to the skeletal SR Ca^{2+} channel protein with high affinity (K_d = 8.6 nM) at the [Ca^{2+}] present in resting muscle (<100 nM), suggesting that the mechanism of CaM interaction with and regulation of the skeletal SR Ca^{2+} channel protein is different from either of the classes of CaM receptors described previously. Although CaM may bind to the skeletal SR Ca^{2+} channel in the presence of EGTA, it is still likely that Ca^{2+} is required for CaM to exert its inhibitory effect on Ca^{2+} release (Smith et al., 1989).

Although it is difficult to accurately determine the stoichiometry of binding of Rh-CaM to the channel protein in the SR vesicles, a reasonable estimate may be made on the basis of the ryanodine-binding activity of the preparations. The functional unit of the channel protein is a tetramer of identical subunits (Lai et al., 1989), each of M_r = 565 000 (Takeshima et al., 1989; Zorzato et al., 1990). One mole of ryanodine specifically binds with high affinity per mole of channel protein tetramer, resulting in a negative cooperative interaction between subunits (Lai et al., 1989). Since our heavy SR preparations averaged 10.6 pmol/mg of [^{3}H]ryanodine-binding activity, this suggests that the channel protein subunit concentration is 42.4 pmol/mg. Scatchard analysis of the Rh-CaM-binding data in the presence of EGTA indicates a single class of CaM-binding sites with a B_{max} of 229 pmol/mg, which is consistent with 5–6 mol of CaM-binding sites per subunit.

In the presence of 0.1 mM Ca^{2+}, there are two classes of binding sites with a B_{max1} for the high-affinity class of CaM-binding sites of 54 pmol/mg, consistent with approximately one CaM-binding site per subunit, and a B_{max2} for the low-affinity class of CaM-binding sites of 166 pmol/mg, corresponding to approximately four CaM-binding sites per subunit.

When 1 mM Mg^{2+} was included with 0.1 mM Ca^{2+}, there was a dramatic shift in the interaction of CaM with the channel protein. The B_{max1} for the high-affinity class of CaM-binding sites was 10 pmol/mg. The Rh-CaM-binding capacity of this class of sites was in close agreement with the [^{3}H]-ryanodine-binding data, suggesting that in the presence of both Ca^{2+} and Mg^{2+} there is approximately 1 mol of CaM bound with high affinity per tetramer. The lower affinity class of sites showed a B_{max2} of 70 pmol/mg, corresponding to approximately two lower affinity Rh-CaM sites per subunit. The effect of inclusion of Mg^{2+} on CaM binding may cause a conformational change in the channel protein in high-affinity CaM-binding sites such that there would be only one high-affinity site per tetramer. Alternatively, in the presence of Mg^{2+}, binding of one CaM to the channel protein may induce a cooperative, allosteric effect on the channel protein, resulting in decreased affinity of the other sites.

These estimates of CaM stoichiometry are based on the affinity-labeling data which suggest that the channel protein is the major receptor for CaM in heavy SR. We cannot exclude the possibility that small amounts of other CaM-binding proteins contribute to the total observed binding. However, the abundance of the channel protein in heavy SR and the limited CaM binding by light SR vesicles (Figure 2) suggest that most of the CaM binding to the SR vesicles is via the channel protein.

These studies provide the first experimental data on the stoichiometry of binding of CaM to the skeletal SR Ca^{2+} channel protein. Previous studies employed sequence analysis to locate potential CaM-binding sites in the channel protein sequence based on the predicted requirement of a basic amphiphilic helix for CaM target proteins (O'Neil & DeGrado, 1990). Takeshima et al. (1989) predicted two CaM-binding sites in the vicinity of residues 3614–3637 and 4295–4325, whereas Zorzato et al. (1990) predicted three different sites at residues 2775–2807, 2877–2898, and 2998–3016. Brandt et al. (1992) reported that CaM inhibits calpain digestion of the skeletal SR channel protein. Analysis of the digestion pattern of the channel protein in the presence and absence of CaM, coupled with the use of computer algorithms to identify consensus sequences for calpain digestion and CaM binding, led to the identification of three more candidate sites for CaM binding: residues 1383–1400, 1974–1996, and 3358–3374. Our experimental data suggest that there may be as many as five to six CaM-binding sites per subunit and that the affinity of each CaM site depends on the divalent metal ion concentration. Our data further indicate that CaM binds to the channel protein under conditions comparable to that of resting muscle (<100 nM $CaCl_2$). This suggests that there may be a different structural element from that of a basic amphiphilic helix for CaM binding in the channel protein, making predictions of CaM-binding domains based on sequence analysis difficult. Experiments are in progress in our laboratory to identify the CaM-binding domains in the channel protein.

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