Voordouw, G., Pollock, W. B. R., Bruschi, M., Guerlesquin, F., Rapp-Giles, B. J., & Wall, J. D. (1990) *J. Bacteriol. 172*, 6122-6126.
Walker, F. A., Huynh, B. H., Scheidt, W. R., & Oswath, S. R. (1986) *J. Am. Chem. Soc. 108*, 5288-5297.
Weber, P. C., Howard, A., Xuong, H. G. H., & Salemme, F. R. (1981) *J. Mol. Biol. 153*, 399-424.
Yagi, T. P., & Ogata, T. (1990) in *Proceedings of the FEMS Symposium Microbiology and Biochemistry of strict anaerobes involved in interspecies hydrogen transfer*, (Belaich, J. P., Bruschi, M., & Garcia, J. L., Eds.) pp 237-248, Plenum Press, New York.

Conformational Changes in the Foot Protein of the Sarcoplasmic Reticulum Assessed by Site-Directed Fluorescent Labeling†

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ABSTRACT: Ca^{2+} release from sarcoplasmic reticulum during excitation–contraction coupling is likely to be mediated by conformational changes in the foot protein moiety of the triadic vesicles. As a preparative step toward the studies of dynamic conformational changes in the foot protein moiety, we have developed a new method that permits specific labeling of the foot protein moiety of the isolated membranes with a fluorophore. A novel fluorescent cleavable photoaffinity cross-linking reagent, sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido)ethyl)dithio)propionate (SAED), was conjugated with site-directing carriers, polylysine (Ca^{2+}-release inducer) and neomycin (Ca^{2+}-release blocker). The conjugates were allowed to bind to polylysine- and neomycin-binding sites of the heavy fraction of SR (HSR). After photolysis, the cross-linked reagent was cleaved by reduction and the fluorescently labeled HSR was separated from the carriers by centrifugation. These procedures led to specific incorporation of the methylcoumarin acetate (MCA) into the foot protein. Polylysine and neomycin bound to different sites of the foot protein, since neomycin, at release-blocking concentrations, did not interfere with polylysine binding. The fluorescence intensity of the foot protein labeled with the carrier, neomycin, showed biphasic changes as a function of ryanodine concentration (increasing up to 1 μM ryanodine and decreasing above it), while with the carrier polylysine, ryanodine induced no change in fluorescence intensity. In contrast, the fluorescence intensity of the foot protein labeled with each of the two carriers, neomycin and polylysine, showed almost identical calcium dependence (first increasing from 0.1 μM to about 3.0 μM calcium concentration, and then decreasing at higher calcium concentrations). These results suggest that modulation of Ca^{2+} release by ryanodine involves a local conformational change in the neomycin-binding region of the foot protein, while that by Ca^{2+} involves conformational changes not only in the neomycin-binding region but also in the polylysine-binding region.

The molecular mechanism of excitation–contraction (e–c) coupling in muscle, especially the mechanism by which transient changes in the T-tubule[1] membrane potential lead to a rapid Ca^{2+} release from the SR, is one of the most important unresolved questions in muscle physiology (Endo, 1977; Martonosi, 1984; Caille et al., 1985; Fleischer & Inui, 1989). Recent studies have unraveled several important molecular components involved in the coupling process. One of the SR proteins with a M_r of approximately 500K is a specific receptor of ryanodine, and it forms a tetrameric complex whose electron microscopic structure is identical to that of an individual foot (the bridge-like structure between the T-tubule and SR; Franzini-Armstrong, 1975; Ferguson et al., 1984; Wagenknecht et al., 1989). This indicates that the ryanodine-binding protein is the major constituent of the foot. The foot protein behaves like a Ca^{2+}-release channel when incorporated into lipid bilayers (Lai et al., 1988; Imagawa et al., 1987; Smith et al., 1986; Hymel et al., 1988), suggesting that the protein responsible for the rapid Ca^{2+} release from SR might be the foot protein. In addition, the α_1 subunit of the dihydropyridine (DHP) receptor located in the T-tubule membrane appears to play a critical role in the mechanism by which the excitation signal elicited in the T-tubule upon stimulation of muscle is

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[1] Abbreviations: SAED, sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido)ethyl)dithio)propionate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); PC, phosphatidylcholine; DACM, *N*-(7-(dimethylamino)-4-methyl-3-coumarinyl)-maleimide; HSR, heavy sarcoplasmic reticulum; DTT, dithiothreitol, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PI, proteolytic enzyme inhibitors; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system.

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Scheme I

Polylysine or Neomycin
+
SAED
⇓ Reacted in the dark for 15 min at 22°
⇓ Quenched by 10X dilution with 10 mM lysine
⇓ Mixed with HSR in the dark
⇓ Centrifuged to remove unreacted SAED and unbound polylysine or neomycin

HSR·[polylysine-SAED] or HSR·[neomycin-SAED]

⇓ photolysis

HSR-SAED-polylysine or HSR-SAED-neomycin

⇓ 100 mM β-ME was added to reduce the disulfide bond
⇓ Centrifuged and dialyzed to remove polylysine or neomycin

HSR-MCA

transmitted to the SR Ca^{2+}-release channel, as indicated by several pieces of evidence. First, the dysgenic mouse incapable of e–c coupling lacks the α_1 subunit (Knudson et al., 1989); second, genetic expression of the α_1 subunit in primary tissue culture of the dysgenic myotubes regenerates e–c coupling and charge movement (Tanabe et al., 1988, 1990; Adams et al., 1990); finally, DHPs block both e–c coupling and charge movement (Hui et al., 1984; Rios & Brum, 1987). Furthermore, the primary structure of this subunit deduced from the cDNA sequence (Tanabe et al., 1987; Catterall, 1988) contains unique sequences that may account for its putative voltage-sensing function. Thus, voltage-sensing by, and charge movement in, the DHP receptor and coupled conformational changes occurring in the foot protein appear to be the key events involved in e–c coupling.

To investigate conformational changes of the foot protein occurring during e–c coupling, we have developed a method that permits specific labeling of the foot protein moiety of the isolated vesicular membranes with a fluorophore. Specific fluorescent labeling of the foot protein was achieved using a novel cleavable, photoactivatable, fluorescent cross-linking reagent recently designed and characterized by Thevenin et al. (1991; details to be published elsewhere). The general scheme for site-directed fluorescent labeling involves, as outlined in Scheme I, derivatization of a carrier with the reagent and photoactivated cross-linking to a recipient-interacting protein, followed by cleavage of the cross-linker which leaves the fluorophore near the binding site of the recipient protein. Thus, site-directed labeling of the foot protein was achieved using the calcium-release inducer, polylysine, and the release blocker, neomycin, as carriers which bind specifically to the foot protein at low concentrations (Cifuentes et al., 1989). The fluorescence intensity of the fluorophore specifically incorporated with these two carriers shows different ryanodine dependence but similar calcium dependence. Considering that neomycin and polylysine appear to have different binding sites on the foot protein, our results suggest that modulation of the calcium channel activity by ryanodine involves a local conformational change, while that by calcium involves more global changes. Thus, the method described here has permitted us to target the fluorescent probe not only to the foot protein moiety of the triad vesicles but also to the specific domains of the protein.

Experimental Procedures

Microsome Preparation. A preparation of heavy sarcoplasmic reticulum (HSR), rich in triad vesicles, was obtained from rabbit skeletal muscles, as previously described (Ikemoto et al., 1989). The final microsomal pellet was resuspended in a solution containing 0.3 M sucrose, protease inhibitors (0.1 mM PMSF, 10 μg/mL aprotinin, 0.8 μg/mL antipain, 2.0 μg/mL soybean trypsin inhibitor), and 20 mM MES, pH 6.8 (PI buffer).

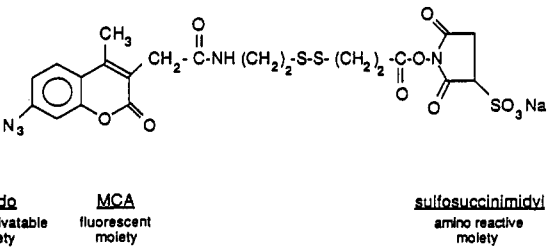

FIGURE 1: Chemical structure of the novel cleavable hetero-bifunctional cross-linking reagent sulfosuccinimidyl 3-((2-(7-azido-4-methylcoumarin-3-acetamido)ethyl)dithio)propionate (SAED).

Calcium-Release Assay. SR vesicles (1 mg/mL) were actively loaded with Ca^{2+} in a solution containing 0.15 M KCl, 0.5 mM MgATP, 50 μM $CaCl_2$, 5.0 mM phosphoenolpyruvate, 10 units/mL pyruvate kinase, 9 μM arsenazo III, and 20 mM MES, pH 6.8 (solution A). Ca^{2+} release was induced by mixing one part of solution A with one part of solution B containing 0.15 M KCl, 4 μg/mL polylysine (M_r = 4000; Sigma), 9 μM arsenazo III, and 20 mM MES, pH 6.8. The effect of neomycin on polylysine-induced Ca^{2+} release was followed after mixing one part of solution A with one part of solution B and various concentrations of neomycin. The time course of the induced Ca^{2+} release was recorded using stopped-flow spectrophotometry, as previously described (Ikemoto et al., 1989; Morii et al., 1986), and the data were analyzed by a computer fit of a single-exponential function to the data.

Site-Directed Fluorescent Labeling of the HSR. A novel cleavable hetero-bifunctional cross-linking reagent SAED (Figure 1) was synthesized [for synthesis and characterization, see Thevenin et al. (1991)]. HSR was labeled with SAED by using two polyamines, polylysine and neomycin, as carriers according to Scheme I. To form polyamine–SAED conjugates, 0.2 mM SAED was incubated with 0.05 mM polylysine (M_r = 4000; Sigma) or 0.4 mM neomycin, in 20 mM HEPES (pH 7.5) for 15 min at 22 °C in the dark. The reaction was quenched by a 10 times dilution with 10 mM lysine. Either 100 μL of polylysine–SAED conjugate (final concentration of polylysine = 0.5 μM) or 50 μL of neomycin–SAED conjugate (final neomycin concentration = 2 μM) was mixed in the dark with 1 mg of HSR protein, brought to 1 mL with PI buffer, and centrifuged for 15 min at 353000*g*. The sedimented fraction was resuspended in 1 mL of PI buffer and photolyzed with UV light in a Pyrex tube at 4 °C for 10 min. β-Mercaptoethanol was added (100 mM final) to cleave the disulfide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged for 15 min at 353000*g*. The sedimented HSR was resuspended in 200 μL of PI buffer at a final protein concentration of 5 mg/mL. As a control, the HSR was treated similarly in the absence of the polyamine carriers.

Gel Electrophoretic Analysis of the Fluorescently Labeled HSR. The MCA-labeled HSR was subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The fluorescence bands on the gel were excited using a UV transilluminator coupled to a 350-nm interference filter and photographed using a 410-nm-cutoff filter. The gels were subsequently stained for protein with Coomassie brilliant blue.

Purification of the Fluorescently Labeled Foot Protein. The fluorescently labeled foot protein was purified on a linear

sucrose gradient after solubilization of the HSR with CHAPS–phosphatidylcholine (PC) buffer according to the method of Lai et al. (1988). Foot protein-enriched fractions were identified by their fluorescence (excitation at 350 nm and emission at 450 nm) with a SPEX Fluorolog fluorometer. To purify the nonfluorescent control foot protein, a fraction of the HSR was labeled with [^{3}H]ryanodine, solubilized, and fractionated on a sucrose gradient, as described above. The purified foot protein fraction was identified by scintillation counting of the gradient fractions. The amount of fluorophore incorporated into the foot protein was estimated from the absorbance at 350 nm of the labeled protein, after subtraction of the absorbance at 350 nm of the unlabeled protein, and using ϵ_{350} = 6450 mol/cm determined in a solution containing 1 M NaCl, 20 mM PIPES (pH = 6.8), 10% sucrose, 0.9% CHAPS, 0.4% PC, and 2% SDS.

Assays of Ryanodine and Calcium-Induced Conformational Changes of the Foot Protein. For the binding of ryanodine to the foot protein, 100 μL of purified foot protein (0.2 mg/mL) was mixed with 1 mL of assay buffer (0.15 M KCl, 2 mM EGTA, 20 mM MES, pH 6.8) containing 1.69 mM $CaCl_2$ (free calcium concentration = 3 μM) and various concentrations of ryanodine. This mixture was incubated at 37 °C for 60 min. To investigate the effective calcium concentration on the fluorescence properties of the foot protein-bound MCA, 100 μL of the purified foot protein (0.2 mg/mL) was mixed with 1 mL of assay buffer containing various concentrations of Ca^{2+}. The fluorescence intensities (excitation at 350 nm and emission at 450 nm) of the samples incubated with ryanodine or calcium were determined in a SPEX Fluorolog fluorometer.

Tritiated Polylysine Binding Assays. Polylysine was tritiated according to the following scheme: 1 mCi (0.021 μmol) [*propionate*-2,3-^{3}H]-*N*-succinimidyl propionate (New England Nuclear/DuPont), whose organic solvent had been evaporated under a stream of nitrogen gas, was added to 0.5 mL of a solution containing 2 mg/mL polylysine (M_r = 4000) and incubated for 4 h at 22 °C. The unbound radioactivity was removed, first by chromatography in a Sephadex G-25 column, and then by dialysis against 0.15 M KCl/20 mM MES (pH 6.8), in a 1000-M_r-cutoff dialysis tubing. The concentration of polylysine was determined with the *o*-phthalaldehyde reagent (Peterson, 1983). To determine the amount of polylysine bound to HSR membranes, HSR (1 mg of protein/mL) was incubated for 5 min with polylysine (75 nM–75 μM) containing 0.080–0.1 μCi/mL [^{3}H]polylysine in 0.5 mL of the assay buffer containing 0.15 M KCl, 10 μM $CaCl_2$, and 20 mM MES (pH = 6.8). The unbound polylysine was removed by centrifugation for 15 min at 353000*g*. The sedimented HSR was rinsed with the above solution and homogenized in 0.5 mL of the same solution. The homogenates were mixed with 10 mL of Ecoscint A for scintillation counting. To determine nonspecific polylysine binding, HSR was incubated with 0.25 mM polylysine and the same amounts of [^{3}H]-polylysine as above, and the radioactivity in the sedimented HSR was counted. The amount of polylysine specifically bound to HSR was calculated from the difference between the total and the nonspecifically bound polylysine.

Results

Specific Fluorescent Labeling of the Foot Protein Moiety of HSR. The cleavable, fluorescent, photoaffinity cross-linker, SAED (for its structure, see Figure 1) was conjugated with two polyamines that have specific effects on Ca^{2+} release: neomycin, which blocks Ca^{2+} release (Palade, 1987), and polylysine, which induces Ca^{2+} release (Cifuentes et al., 1989).

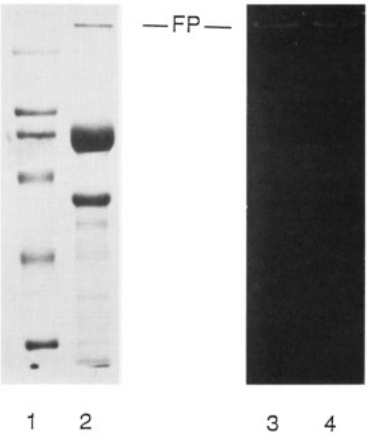

FIGURE 2: SDS–polyacrylamide gel electrophoretic analysis of the fluorescently labeled proteins of HSR. The HSR was first labeled using either SAED–polylysine or SAED–neomycin as carriers, as described in Experimental Procedures. The proteins were then separated on a 5–15% gradient SDS–polyacrylamide gel. Lane 1: molecular weight reference proteins (M_r 200K, 110K, 97K, 66K, 45K, 29K). Lane 2: Coomassie brilliant blue-stained HSR (50 μg of protein). Lane 3: fluorescently labeled HSR (100 μg) using polylysine as a carrier. Lane 4: fluorescently labeled HSR (100 μg) using neomycin as a carrier. FP, foot protein.

Conjugation was made via reaction of the succinimidyl moiety of SAED with amino groups of neomycin and polylysine. The fluorescent moiety of the cross-linker, MCA, was incorporated into the neomycin- and polylysine-binding sites of HSR according to the procedures outlined in Scheme I. Briefly, the fluorescently labeled polyamines were bound to HSR in the dark and covalently cross-linked by photolysis. Then, the cross-linked products were cleaved with β-mercaptoethanol and the HSR was washed. The resulting HSR preparation was selectively labeled in the foot protein moiety, as shown in Figure 2. There is a faint fluorescent band with a molecular weight just below that of the foot protein. This presumably represents a partially cleaved foot protein peptide. In a control labeling with SAED but without carriers, no appreciable fluorescent labeling of any protein component of HSR was detected. Similarly, there was no detectable fluorescent labeling when the HSR was incubated with the SAED–carrier conjugate without photolysis followed by washing in a reducing solution.

The fluorescently labeled foot protein was purified from HSR according to Lai et al. (1988). The stoichiometry of MCA incorporation was determined after purification of the labeled foot protein. Under our standard conditions of incorporation (2 μM neomycin–SAED or 0.5 μM polylysine–SAED reacted with 1 mg/mL HSR protein), 0.75 mol and 1.15 mol of MCA were incorporated/mol of the 565 223-kDa foot protein (Takeshima et al., 1989), respectively. On the other hand, the amount of the carrier polylysine that remained bound with the purified foot protein was 2 pmol/mg or 1.1 mmol/mol of foot protein, as determined by labeling HSR with the [^{3}H]polylysine–SAED conjugate followed by treatment with β-mercaptoethanol and purification of the foot protein.

The Two Carriers, Polylysine and Neomycin, Bind to Different Sites of the Foot Protein. Polylysine, at low concentrations used for incorporation of MCA into the foot protein (0.5 μM), induces Ca^{2+} release from sarcoplasmic reticulum (Figure 3A). This Ca^{2+} release was blocked by increasing concentrations of neomycin (Figure 3A), with a half-maximal inhibition at about 0.2 μM (Figure 3B). These results suggest that the two carriers used for site-directed incorporation of MCA into the foot protein have definitive effects on Ca^{2+} release function; viz., polylysine is a Ca^{2+}-release inducer [cf.

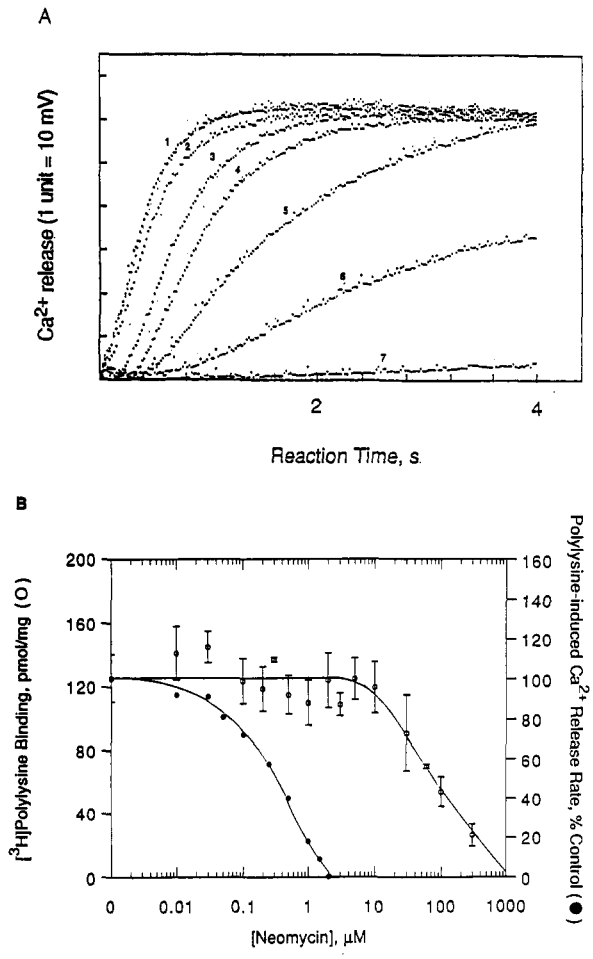

FIGURE 3: Neomycin concentration dependence of polylysine-induced Ca^{2+} release and [^{3}H]polylysine binding to HSR. (A) Time course of Ca^{2+} release induced by polylysine in the presence of various concentrations of neomycin (from curve 1 to 7: 0, 0.5, 0.1, 0.25, 1, 1.5, and 2 μM). A total of 1 mg/mL HSR was actively loaded with Ca^{2+}, and Ca^{2+} release was induced by 0.5 μM polylysine (M_r 4000), as described in Experimental Procedures. (B) [^{3}H]Polylysine binding (O) and polylysine-induced Ca^{2+} release (●) in the presence of various concentrations of neomycin. HSR was incubated with 0.5 μM [^{3}H]polylysine in the presence of various concentrations of neomycin, and the amounts of polylysine bound were determined by scintillation counting of the sedimented HSR (see Experimental Procedures). The initial rates of Ca^{2+} release (Ak) were calculated by fitting a single-exponential function of [$y = y_0 + A(1 - e^{-kt})$] to the stopped-flow curves. Each point of polylysine-binding data represents the average ± standard deviation (n = 3); each point of Ca^{2+}-release data represents the average of nine experiments.

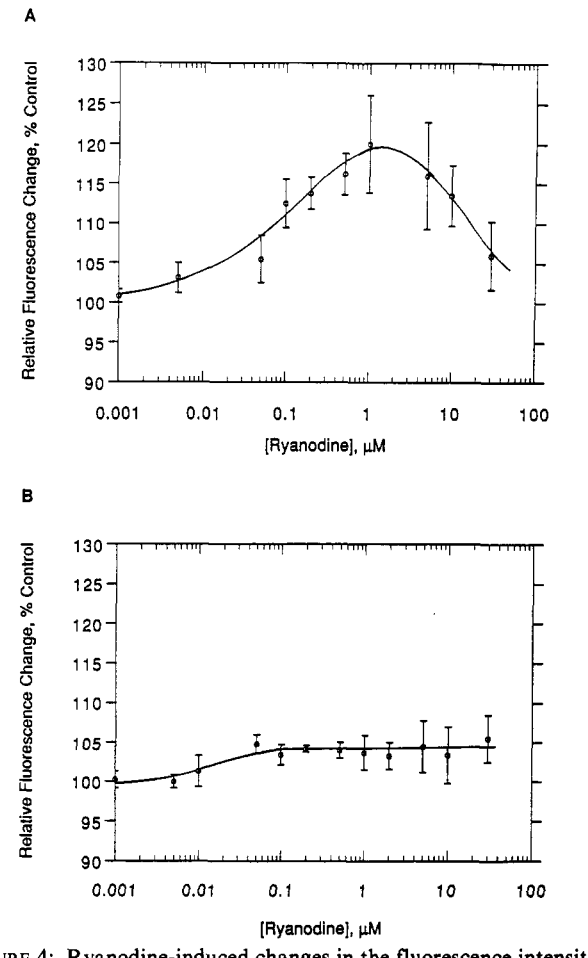

FIGURE 4: Ryanodine-induced changes in the fluorescence intensity of MCA incorporated into the foot protein by mediation of neomycin (A) and polylysine (B). Purified, fluorescently labeled foot protein was resuspended in a solution containing different concentrations of ryanodine, and fluorescence intensity was determined. The change in fluorescence intensity relative to no ryanodine, ΔF, was calculated after subtraction of the background intensity of a solution containing the same concentration of purified, unlabeled foot protein.

Cifuentes et al. (1989)] and neomycin is a Ca^{2+}-release blocker [cf. Palade (1987)].

Figure 3B illustrates that the amount of [^{3}H]polylysine bound to HSR at 0.5 μM polylysine is virtually unaffected by neomycin in the concentration range up to 10 μM, in which polylysine-induced Ca^{2+} release is blocked completely. This argues against competitive binding of neomycin to the polylysine-binding site on the foot protein and suggests that at the concentrations of the two carriers used for probe incorporation (0.5 μM polylysine and 2 μM neomycin) the two carriers bound at two different sites on the protein. At higher concentrations of neomycin (>10 μM), however, the amount of polylysine bound sharply decreased, suggesting that neomycin at its high concentration becomes competitive to the polylysine-binding site(s).

Ryanodine-Induced Conformational Changes of the Purified Foot Protein. In an attempt to examine conformational changes in the foot protein induced by ryanodine, the foot protein labeled with either neomycin or polylysine as the carrier was purified, and the ryanodine concentration dependence of the fluorescence intensity of the incorporated probe was investigated. As shown in Figure 4A, the fluorescence incorporated into the foot protein with neomycin first increased ($\Delta F/F_0$ = 21 ± 6%, n = 9) in a range of ryanodine concentration from 1 nM to 1 μM which activates Ca^{2+} efflux from SR (Fleischer et al., 1985; Lattanzio et al., 1987; Meissner, 1986). Above 1 μM ryanodine, it is inhibitory to Ca^{2+} efflux [cf. Chu et al. (1988), Lattanzio et al. (1987), Meissner (1986), and Seiler et al. (1984)] and the fluorescence intensity decreased. These observations suggest that the biphasic nature of the ryanodine dependence of the incorporated fluorescence represents conformational changes of the foot protein corresponding to ryanodine-induced activation and inhibition of the Ca^{2+} channel.

In contrast with the above observations, the fluorescence incorporated into the foot protein with polylysine showed virtually no ryanodine concentration dependence (Figure 4B), suggesting that the two carriers deliver the fluorescent probe to two different loci on the foot protein as predicted from the experiments shown in Figure 3. Taken together, we propose that ryanodine-induced conformational changes of the foot protein are limited to the neomycin-binding locus and do not extend to the polylysine-binding region.

Calcium-Induced Conformational Changes of the Purified

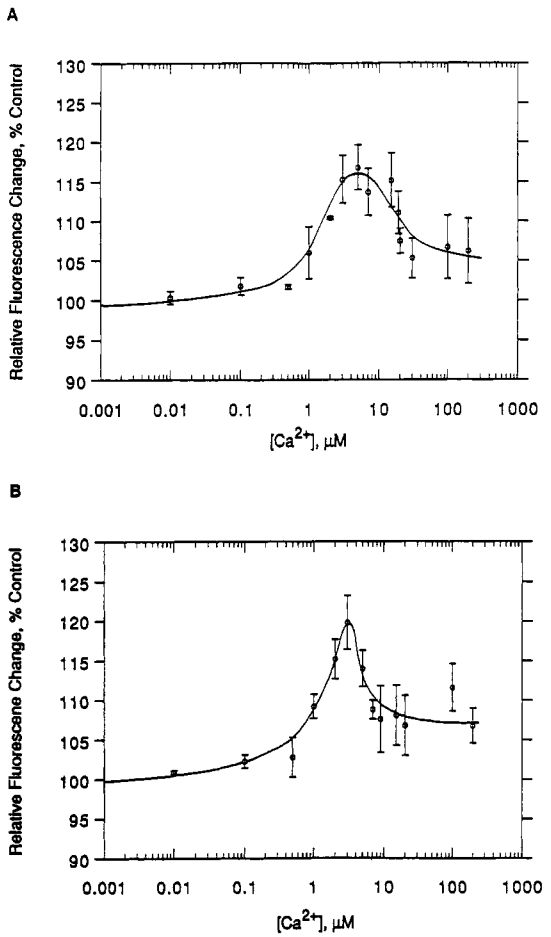

FIGURE 5: Ca^{2+} dependence of the fluorescence intensity of the MCa incorporated into the foot protein by mediation of neomycin (A) and polylysine (B). Purified, fluorescently labeled foot protein was resuspended in solutions of various calcium concentrations adjusted by EGTA–calcium buffers, and the fluorescence intensity of the protein-bound MCA was determined. ΔF was corrected for background, as described in the Experimental Procedures. Each data point represents the average ± standard deviation (n = 15).

Foot Protein. The fluorescence of the labeled foot protein showed a similar Ca^{2+} dependence regardless of the type of carrier used for incorporation (Figure 5; panel A, neomycin-mediated incorporation, panel B, polylysine-mediated incorporation). The MCA fluorescence sharply increased ($\Delta F/F_0$ = 17.5 ± 3.0%, n = 15, Figure 5A; $\Delta F/F_0$ = 20 ± 3.4%, n = 15, Figure 5B) with Ca^{2+} concentrations from 0.1 μM to about 3.0 μM and decreased at higher Ca^{2+} concentrations (Figure 5). The fluorescence decrease in Figure 5B (MCA incorporation with polylysine) occurs at a much lower Ca^{2+} concentration than in Figure 5A (MCA incorporation with neomycin). However, the overall profile of the Ca^{2+} concentration dependence is essentially identical in both cases; this is in sharp contrast to the fact that ryanodine produces virtually no fluorescence change when the MCA probe is incorporated with the polylysine carrier. The biphasic Ca^{2+} concentration dependence of fluorescence was similar to that of calcium-induced activation and inhibition of Ca^{2+} release (Edno, 1977; Miyamoto & Racker, 1982; Kim et al., 1983; Ikemoto et al., 1985; Meissner et al., 1986). Thus, the incorporated fluorophore could be reporting calcium-induced conformational changes of the foot protein corresponding to the calcium-induced activation and inhibition of the channel. The similarity of the Ca^{2+} dependence of fluorescence incorporated with both carriers suggests that Ca^{2+}-induced conformational changes take place not only in the neomycin-binding region but also in the polylysine-binding region.

Discussion

As described in the introduction, recent studies have resolved two important molecular components involved in the mechanism of e–c coupling by which transient changes in the T-tubule membrane potential lead to Ca^{2+} release from SR: viz., the DHP receptor (a voltage-sensing component of the T-tubule membrane) and the foot protein (a large M_r protein of SR responsible for the interaction with the T-tubule and Ca^{2+} release from SR as well). Thus, the most plausible hypothesis is that the DHP receptor would sense the changes in the T-tubule membrane potential and produce an excitation signal (e.g., nonlinear charge movement; Schneider & Chandler, 1973; Hui et al., 1984; Rios & Brum, 1987); the signal is transmitted to SR, which produces conformational changes in the foot protein, leading to the activation of the Ca^{2+}-release channel located in the protein. As shown in our recent studies with the isolated foot protein labeled with the fluorescent probe DACM (Ohkusa et al., 1991), the fluorescence intensity of the foot protein-attached probe changed upon the binding of various effectors of Ca^{2+} release, suggesting that conformational changes of the foot protein are involved in the process of activation of the Ca^{2+}-release channel. Thus, it is anticipated that the excitation signal transmitted from the T-tubule would produce conformational changes of the foot protein, which in turn activates the Ca^{2+}-channel.

To investigate the conformational changes occurring in the foot protein during e–c coupling, one must use intact triad vesicles, and it is essential to establish the method that permits specific fluorescent labeling of the foot protein moiety out of the numerous other proteins present in the vesicular preparation. As shown in previous studies (Thevenin et al., 1991), a three-step procedure—(i) conjugation of the fluorescent hetero-bifunctional agent, SAED, with a carrier such as trypsin inhibitor that binds specifically to trypsin; (ii) cross-linking of the conjugated compound (SAED–trypsin inhibitor) with the target protein, trypsin; (iii) removal of the carrier, trypsin inhibitor, from trypsin—resulted in the transfer of the fluorescent moiety of SAED, MCA, to trypsin. The same principle and method must allow one to incorporate the fluorescent probe, MCA, into the foot protein moiety of the triad vesicle in a site-directed fashion, since several suitable carriers are available, such as polylysine that binds specifically to the foot protein moeity of SR vesicles to induce Ca^{2+} release (Cifuentes et al., 1989).

One of the most important aspects of this paper is that the combined use of the novel fluorescent cleavable hetero-bifunctional cross-linker, SAED, and the foot protein-specific carriers neomycin and polylysine has permitted specific fluorescent labeling of the foot protein moeity of the HSR out of the numerous other proteins present in this vesicular system. Furthermore, the two types of carriers have mediated differential labeling of two distinct regions of the protein, as evidenced by the fact that the effects of ryanodine on the fluorescence intensity of the foot protein-attached probe were different depending upon the types of carrier used (cf. Figure 4A,B). Thus, the method described here has permitted us to target the fluorescent probe not only to a particular protein but also to the specific domains of the protein.

The carriers used for probe incorporation are the well-characterized effectors of Ca^{2+} release: viz., polylysine, Ca^{2+}-release inducer (Cifuentes, 1989), and neomycin, Ca^{2+}-release blocker (Palade, 1987; this study). In other words, the regions of the foot protein that are labeled using these carriers are functionally meaningful regions; viz., the

region labeled by mediation of polylysine and that by neomycin would present a release-triggering domain and a release-blocking domain, respectively. Consequently, the present finding that ryanodine affects only the neomycin-mediated MCA suggests that ryanodine produces a local conformational change of the foot protein in the vicinity of the blocking domain. On the other hand, the finding that Ca^{2+} produces conformational changes in both triggering and blocking domains suggests that the regulation by Ca^{2+} of the foot protein involves conformational changes in both the release-triggering and release-blocking domains. Importantly, as shown in the dependence on the concentration of both ryanodine and Ca^{2+}, the increase of the MCA fluorescence correlates well with activation of Ca^{2+} channel activity and Ca^{2+} efflux by these agents, while the decreases in fluorescence are correlated with suppression of these activities. This suggests that the fluorescence response of the protein-bound MCA described here represents the conformational change of the foot protein relevant to the mechanism by which Ca^{2+} channels are regulated.

In conclusion, the conjugates of the novel fluorescent cleavable photoaffinity cross-linking reagent, SAED, with the two foot protein-specific carriers (polylysine and neomycin) have permitted us to incorporate the MCA fluorophore of the reagent into the foot protein moiety of triad vesicle in a site-directed fashion. Furthermore, the use of different types of carrier permitted differential labeling of two distinct regions of the foot protein. Fluorescence assays of the labeled foot protein at various concentrations of ryanodine and Ca^{2+} suggested that the regulation of Ca^{2+} channel function by ryanodine is mediated by conformational changes occurring in the neomycin-binding domain, and regulation by Ca^{2+} is mediated by those occurring in both neomycin- and polylysine-binding domains. The present study opens new possibilities for further studies of the role of the foot protein in e–c coupling. For example, the established capability of incorporating the fluorescent probe into the selected domains of the foot protein would permit the investigation of dynamic conformational changes occurring in these domains during SR Ca^{2+}-release induced by depolarization of the T-tubule. It would also be possible to identify the locations of various functionally distinct domains (e.g., release-triggering domain and release-blocking domain) within the primary structure of the foot protein by sequencing the fluorescently labeled peptides. Preliminary experiments on both of these aspects are underway.

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References

Adams, B. A., Tanabe, T., Mikami, A., Numa, S., & Beam, K. G. (1990) *Nature 346*, 569–572.

Caille, J., Ildefonse, M., & Rougier, O. (1985) *Prog. Biophys. Mol. Biol. 46*, 185–239.

Catterall, W. A. (1988) *Science 242*, 50–61.

Chu, A., Sumbilla, C., Scales, D., Piazza, A., & Inesi, G. (1988) *Biochemistry 27*, 2827–2833.

Cifuentes, M. E., Ronjat, M., & Ikemoto, N. (1989) *Arch. Biochem. Biophys. 273*, 554–561.

Dixon, D., Brandt, M., & Haynes, D. H. (1984) *J. Biol. Chem. 259*, 13737–13741.

Endo, M. (1977) *Physiol. Rev. 57*, 71–108.

Ferguson, D. G., Schwartz, H. W., & Franzini-Armstrong, C. (1984) *J. Cell Biol. 99*, 1735–1742.

Fleischer, S., & Inui, M. (1989) *Annu. Rev. Biophys. Chem. 18*, 333–364.

Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., & Fleer, E. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7256–7259.

Franzini-Armstrong, C. (1975) *Fed. Proc. 34*, 1382–1389.

Hui, C. S., Milton, R. L., & Eisenberg, R. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2582–2585.

Hymel, L., Inui, M., Fleischer, S., & Schindler, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 441–445.

Ikemoto, N., Antoniu, B., & Kim, D. H. (1984) *J. Biol. Chem. 259*, 13151–13158.

Ikemoto, N., Antoniu, B., & Meszaros, L. G. (1985) *J. Biol. Chem. 260*, 14096–14100.

Ikemoto, N., Ronjat, M., Meszaros, L. G., & Koshita, M. (1989) *Biochemistry 28*, 6764–6771.

Imagawa, T., Smith, J. S., Coronado, R., & Campbell, K. (1987) *J. Biol. Chem. 262*, 16636–16643.

Kim, D.-H., Ohnishi, S. T., & Ikemoto, N. (1983) *J. Biol. Chem. 258*, 9662–9668.

Knudson, C. M., Chaudhari, N., Sharp, A. H., Power, J. A., Bean, K. G., & Campbell, K. (1989) *J. Biol. Chem. 264*, 1345–1348.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lai, F. A., Erickson, H. P., Block, B. A., & Meissner, G. (1988) *Nature 331*, 315–320.

Lattanzio, F. A., Schlatterer, R. G., Nicaar, M., Campbell, K., & Sutko, J. (1987) *J. Biol. Chem. 262*, 2711–2718.

Martonosi, A. N. (1984) *Physiol. Rev. 64*, 1240–1320.

Meissner, G. (1986) *J. Biol. Chem. 261*, 6300–6306.

Meissner, G., Darling, E., & Eveleth, J. (1986) *Biochemistry 25*, 236–244.

Miyamoto, H., & Racker, E. (1982) *J. Membr. Biol. 66*, 193–201.

Morri, M., Danko, S., Kim, D. H., & Ikemoto, N. (1986) *J. Biol. Chem. 261*, 2343–2348.

Ohkusa, T., Kang, J. J., Morri, M., & Ikemoto, N. (1991) *J. Biochem. 109*, 609–615.

Palade, P. (1987) *J. Biol. Chem. 262*, 6149–6154.

Peterson, G. L. (1983) *Methods Enzymol. 91*, 95–119.

Rios, E., & Brum, G. (1987) *Nature 325*, 717–720.

Schneider, M. F., & Chandler, W. K. (1973) *Nature 242*, 244–246.

Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., & Jones, L. R. (1984) *J. Biol. Chem. 259*, 8550–8557.

Smith, J. S., Coronado, R., & Meissner, G. (1986) *J. Gen. Physiol. 88*, 573–588.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., & Numa, S. (1989) *Nature 339*, 439–445.

Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T., & Numa, S. (1987) *Nature 328*, 313–318.

Tanabe, T., Beam, K. G., Powell, J. A., & Numa, S. (1988) *Nature 336*, 134–139.

Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T. T., & Numa, S. (1990) *Nature 346*, 567–569.

Thevenin, B. J.-M., Shahrokh, Z., Willard, R. L., Fujimoto, E. K., Ikemoto, N., & Shohet, S. B. (1991) *Biophys. J. 59*, 358a.

Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M., & Fleischer, S. (1989) *Nature 338*, 167–170.