Dual Regulation of the Skeletal Muscle Ryanodine Receptor by Triadin and Calsequestrin†

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ABSTRACT: Triadin, a calsequestrin-anchoring transmembrane protein of the sarcoplasmic reticulum (SR), was successfully purified from the heavy fraction of SR (HSR) of rabbit skeletal muscle with an anti-triadin immunoaffinity column. Since depletion of triadin from solubilized HSR with the column increased the [^{3}H]ryanodine binding activity, we tested a possibility of triadin for a negative regulator of the ryanodine receptor/Ca^{2+} release channel (RyR). Purified triadin not only inhibited [^{3}H]ryanodine binding to the solubilized HSR but also reduced openings of purified RyR incorporated into the planar lipid bilayers. On the other hand, calsequestrin, an endogenous activator of RyR [Kawasaki and Kasai (1994) *Biochem. Biophys. Res. Commun. 199*, 1120−1127; Ohkura et al. (1995) *Can. J. Physiol. Pharmacol. 73*, 1181−1185] potentiated [^{3}H]ryanodine binding to the solubilized HSR. Ca^{2+} dependency of [^{3}H]ryanodine binding to the solubilized HSR was reduced by triadin, whereas that was enhanced by calsequestrin. Interestingly, [^{3}H]ryanodine binding to the solubilized HSR potentiated by calsequestrin was reduced by triadin. Immunostaining with anti-triadin antibody proved that calsequestrin inhibited the formation of oligomeric structure of triadin. These results suggest that triadin inhibits the RyR activity and that RyR is regulated by both triadin and calsequestrin, probably through an interaction between them. In this paper, triadin has been first demonstrated to have an inhibitory role in the regulatory mechanism of the RyR.

The ryanodine receptor (RyR)[1] is the Ca^{2+} release channel in the sarcoplasmic reticulum (SR), which plays a key role in skeletal muscle excitation−contraction coupling (*1*, *2*). The plant alkaloid ryanodine binds with high affinity to the RyR in an open state to cause inactivation of Ca^{2+} release from the SR (*3*, *4*). Therefore, ryanodine has been extensively used as a beneficial biochemical probe to analyze the RyR function (*5*). The RyR has been purified (*6*−*8*) and extensively investigated, including reconstitution into planar lipid bilayers (*6*, *8*), cDNA cloning (*9*), functional expression (*10*), and electron microscopic studies (*2*, *11*).

Several proteins including calmodulin (*12*, *13*) and FKBP12 (*14*, *15*) are reported to regulate the RyR from the cytoplasmic side. While calsequestrin, which is a major protein in the lumen of the SR for Ca^{2+} binding with moderate affinity and high capacity (*16*), is anchored to the luminal face of the junctional SR and is thought to sequester and concentrate Ca^{2+} near the RyR (*17*). Luminal Ca^{2+} in the SR is recognized to be important in regulating the RyR, for the RyR-mediated Ca^{2+} release is related to Ca^{2+}-induced conformational changes of calsequestrin (*18*, *19*). Decreasing luminal Ca^{2+} in the SR to 100 μM was shown to cause a significant inhibition of Ca^{2+} release induced by caffeine or ATP (*18*). Recently, it has been reported that adding calsequestrin to the luminal side of the SR directly evoked potentiation of channel current mediated through the RyR (*20*, *21*). Myotoxin *a*, a novel type Ca^{2+}-releasing agent that induces Ca^{2+} release from the heavy fraction of SR (HSR) without binding to the RyR, was also shown to require calsequestrin to produce its effect (*21*, *22*).

Triadin is a transmembrane glycoprotein in the junctional SR, colocalizing with the RyR (*23*−*25*). Triadin was shown to link the dihydropyridine receptor α_1-subunit to the RyR (*26*, *27*). Triadin has attracted attention as a candidate protein for playing an important role in excitation−contraction

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[1] Abbreviations: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; HSR, heavy fraction of sarcoplasmic reticulum; JFM, junctional face membrane; PAGE, polyacrylamide gel electrophoresis; $C_{12}E_9$, poly(oxyethylene 9-lauryl ether); MOPS, 3-(*N*-morpholino)propanesulfonic acid; p-APMSF, (*p*-amidino)phenylmethanesulfonyl fluoride.

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coupling, since a monoclonal antibody raised against triadin inhibited the slow phase of depolarization-induced Ca^{2+} release from the SR (*28*). More recently, triadin has been reported to bind to calsequestrin and the RyR in a Ca^{2+}-dependent manner (*29*). These findings strengthen the hypothesis that triadin is regulating the RyR, receiving signals not only from the dihydropyridine receptor but also from calsequestrin perceiving the luminal Ca^{2+} concentration in the SR.

Here, we first report the direct effects of purified triadin and calsequestrin on the RyR function. Our results indicate that the activity of RyR is inhibited by triadin and is potentiated by calsequestrin and that these opposite effects are probably produced by their interaction. We propose a dual regulatory mechanism of the RyR by triadin and calsequestrin in skeletal muscle SR.

MATERIALS AND METHODS

Materials. Peptides corresponding to amino acid residues 3−16, 25−38, and 33−46 of triadin were synthesized by Takara Shuzo (Shiga, Japan). Anti-rabbit IgG antibody conjugated with alkaline phosphatase and Immunlite II (immunostaining assay kit for enhanced chemiluminescence) were from Bio-Rad (Richmond, CA). [^{3}H]Ryanodine (2.62 MBq/μmol) was from DuPont NEN (Boston, MA). Ryanodine was from S. B. Penick (New York). Protein A−Sepharose 4B was from Zymed (San Francisco, CA). Phenyl-Sepharose CL-4B was from Pharmacia Biotech (Uppsala, Sweden). Formyl-Cellulofine was from Seikagaku Kogyo (Tokyo, Japan). Poly(oxyethylene ether) W-1 (substitute for Lubrol PX), poly(oxyethylene 9-lauryl ether) ($C_{12}E_9$), soybean lecithin (type IV-S), and heparin−agarose were from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Preparation of the HSR and the Junctional Face Membrane-Attached or -Depleted Calsequestrin. The HSR was prepared from rabbit skeletal muscle by the method of Kim et al. (*30*) with minor modifications. White muscle was homogenized in 5 volumes of 5 mM Tris−maleate (pH 7.0) and centrifuged at 5000*g* for 15 min. The supernatant was further centrifuged at 12000*g* for 30 min. The pellet was suspended in a solution containing 90 mM KCl and 5 mM Tris−maleate (pH 7.0) and centrifuged at 70000*g* for 40 min. The HSR pellet was resuspended in the same solution, quickly frozen in liquid nitrogen, and stored at −80 °C until use. The junctional face membrane (JFM) was prepared by the method of Costello et al. (*31*) with modifications. The HSR was preincubated with a solution containing 10% sucrose and 5 mM HEPES−NaOH (pH 7.4) and centrifuged at 100000*g* for 30 min. The HSR pellet was suspended with the above buffer to set the protein concentration to 3.3 mg/mL and treated with 1 mM $CaCl_2$ for 10 min, followed by treatment with 0.5% poly(oxyethylene ether) W-1 for 20 min. The treated HSR was centrifuged at 100000*g* for 30 min and the obtained pellet, which contained calsequestrin abundantly, was suspended again in the above buffer. This suspension was centrifuged at 100000*g* for 30 min, following treatment for 10 min in the presence or absence of 2 mM EGTA. The pellet obtained by pretreatment with or without EGTA was named as calsequestrin-attached JFM or calsequestrin-depleted JFM, respectively. Both pellets were stored on ice in the final suspension solution for the HSR preparation and used within 3 days. All preparations were carried out in the presence of 76.8 μM aprotinin and 0.83 mM benzamidine.

Purification of the RyR. The RyR was purified by the method of Inui et al. (*7*) and Lai et al. (*8*) with modifications. HSR (3 mg/mL) was suspended in 4% CHAPS, 2% soybean lecithin, 1 M NaCl, 20 mM Tris-HCl (pH 7.4), 2 μg/mL leupeptin, and 2 mM DTT. The suspension was placed on ice for 10 min and then centrifuged at 220000*g* for 30 min. The supernatant was separated by centrifugation at 100000*g* for 16 h with a linear gradient of 5−20% sucrose in 1% CHAPS, 0.5% soybean lecithin, 1 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 μg/mL leupeptin, and 2 mM DTT. The fractions corresponding to the sucrose concentration of 12.5−20% were collected and diluted in 0.3 M sucrose, 1% CHAPS, 0.5% soybean lecithin, 0.1 M NaCl, 20 mM Tris-HCl (pH 7.4), 1 μg/mL leupeptin, and 2 mM DTT. The above diluted fractions were loaded on a heparin−agarose (1.5 × 5.8 cm) column preequilibrated with the above solution containing 0.1 M NaCl. The column was washed with the same solution and eluted with a linear gradient between 0.1 and 0.8 M NaCl consisting of the same solution except the NaCl concentration. On the basis of the profile of each fraction checked by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), the RyR- (∼400 kDa) rich fraction was pooled and concentrated with a Vivapore 10-mL concentrator (Vivascience, Lincoln, U.K.) and then dialyzed against 0.3 M sucrose, 1 M KCl, 20 mM Tris-HCl (pH 7.4), 0.5 μg/mL leupeptin, and 1 mM DTT. The purified RyR was quickly frozen in liquid nitrogen and stored at −80 °C until use.

Purification of Calsequestrin. Calsequestrin was purified as described previously (*32*). HSR (1 mg/mL) was treated with 100 mM Na_2CO_3 (pH 11.4) for 30 min, followed by centrifugation at 100000*g* for 30 min. The carbonate supernatant fraction was set to 0.5 M NaCl, 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)−Tris (pH 7.0), and 1 mM DTT and then applied to a phenyl-Sepharose (1.5 × 4 cm) column, preequilibrated with a solution consisting of 0.5 M NaCl, 0.1 mM EGTA, 10 mM MOPS−Tris (pH 7.4), and 1 mM DTT. The column was washed with the same solution and eluted with the same solution plus 10 mM $CaCl_2$. On the basis of the profile of each fraction checked by SDS−PAGE, the calsequestrin- (63 kDa) rich fraction was pooled and dialyzed against 1 M KCl and 5 mM Tris-HCl (pH 7.4). Purified calsequestrin was quickly frozen in liquid nitrogen and stored at −80 °C until use.

Production and Purification of Anti-Triadin Polyclonal Antibody. Anti-triadin polyclonal antibody was produced in rabbits according to standard protocols against a synthetic peptide corresponding to rabbit skeletal muscle triadin sequence (14 amino acid residues, 33−46) (*25*), KRTVT-EDLVTTFSS-C. A cysteine was added to the C terminus for linking to porcine thyroglobulin via lysines using the bifunctional agent *m*-maleimidobenzoyl *N*-hydroxysuccinimide ether. To purify the antibody IgG, the collected antiserum was precipitated by 2 M ammonium sulfate and suspended in 5 mM potassium phosphate buffer (pH 8.0) containing 0.5 M NaCl, followed by dialysis against the same solution. The dialyzed suspension was applied to a DEAE-cellulose column (Whatman, Maidstone, U.K.) preequilibrated with the solution. The IgG fraction, obtained as the

flowthrough, was dialyzed against 50 mM sodium phosphate buffer (pH 7.2), quickly frozen in liquid nitrogen, and stored at −80 °C until use. Preimmune IgG was also prepared from the same rabbits before immunization with the synthetic peptide antigen. Preabsorbed antibody was obtained from the supernatant of IgG fraction incubated with the conjugate of the synthetic peptide of triadin and thyroglobulin. In some experiments, the IgG was digested with papain to obtain the Fab fraction. IgG (20 mg/mL) was incubated for 4 h at 37 °C with 0.2 mg/mL papain in 5 mM sodium phosphate buffer (pH 8.0) containing 10 mM cysteine and 2 mM EDTA. After the incubation, the digested IgG was dialyzed against 50 mM sodium phosphate buffer (pH 7.2) and applied to a protein A−Sepharose (1.5 × 0.57 cm) column, preequilibrated with the same buffer. The IgG Fab fraction, obtained from the flowthrough, was quickly frozen in liquid nitrogen and stored at −80 °C until use.

Preparation of Anti-Triadin Affinity Column and Purification of Triadin. Anti-triadin affinity column was prepared as follows. One gram (wet) of formyl-Cellulofine gel was thoroughly washed with water and preequilibrated with 50 mM sodium phosphate buffer (pH 7.2). Then the buffer was removed and the gel was incubated with 3 mg/mL anti-triadin polyclonal antibody IgG for 6 h. At the incubation time of 2 h, 5 mg of $NaCNBH_3$ was added to reduce Schiff bases. After the incubation, the reaction solution was discarded and the gel linked with the antibody IgG was blocked for 3 h by 50 mM sodium phosphate buffer (pH 7.2) including 0.1 M ethanolamine. Prepared anti-triadin antibody column was used for purification of triadin. For a particular experiment, preimmune IgG-linked column was also prepared as described above except with the use of preimmune IgG instead of anti-triadin IgG. Triadin was purified as follows. HSR (1 mg/mL) was suspended in 1% poly(oxyethylene ether) W-1, 0.1% soybean lecithin, 1 M KCl, 5 mM Tris-HCl (pH 7.4), 1 μg/mL leupeptin, and 0.1 mM *p*-amidinophenylmethanesulfonyl fluoride (*p*-APMSF). The suspension was placed on ice for 10 min and then centrifuged at 120000*g* for 1 h. One milliliter of the supernatant was applied three times to the anti-triadin antibody column, preequilibrated with a solution consisting of 0.2% $C_{12}E_9$, 0.02% soybean lecithin, 1 M KCl, 5 mM Tris-HCl (pH 7.4), 1 μg/mL leupeptin, and 0.3 mM *p*-APMSF. The flowthrough fraction was named as triadin-depleted solubilized HSR, because the column had trapped triadin. The column was washed with the solution used for preequilibration, and triadin was eluted with 100 mM glycine hydrochloride (pH 3.0) and immediately neutralized. Purified triadin was dialyzed against 1 M KCl and 5 mM Tris-HCl (pH 7.4), quickly frozen by liquid nitrogen, and stored at −80 °C until use. With almost the same procedures using the column-linked preimmune IgG, instead of anti-triadin IgG, triadin-retained solubilized HSR was obtained as a flowthrough and used for a particular experiment. Heat denaturation of purified triadin was performed by the incubation of triadin at 100 °C for 30 min. Reduced triadin was obtained by incubation with β-mercaptoethanol (1 M).

Immunostaining with Anti-Triadin Antibody. SDS−PAGE was performed with the buffer system of Laemmli (*33*) with a 7.5% acrylamide gel. Gels were stained by Coomassie Brilliant Blue or silver. The separated proteins were electrophoretically transferred onto PVDF membranes at 12 V for 3 h in the presence of 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. The membranes were washed four times by TBS-T [Tris-buffered saline (0.3 M NaCl and 20 mM Tris-HCl (pH 7.5)) containing 0.05% Tween-20] and blocked overnight by a blocking buffer (20 mg/mL BSA dissolved in TBS). After washing in TBS-T, the membranes were incubated for 4 h with 100-fold diluted anti-triadin antibody. The membranes were washed again in TBS-T, and then the bound anti-triadin antibody was detected with alkaline phosphatase-conjugated anti-rabbit IgG and visualized chemiluminescently with using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]-decan}-4-yl)phenyl phosphate as a substrate.

[^{3}H]Ryanodine Binding Assay. The HSR (0.1 mg/mL), its fractionated samples (0.05−0.2 mg/mL), or the purified RyR (1 μg/mL) was incubated with 10 nM [^{3}H]ryanodine for 2 h at 37 °C in a binding buffer [0.3 M sucrose, 1 M KCl, $CaCl_2$ equivalent to 316 nM−1 mM free Ca^{2+}, 20 mM Tris-HCl (pH 7.4), 0.1% soybean lecithin, and 0.1 mM *p*-APMSF] in a total volume of 500 μL. Poly(ethylene glycol) (1%) was added at the incubation time of 90 min. The total binding was measured by filtering aliquots of the samples through poly(ethylenimine)-treated Whatman GF/B glass filters. The radioactivity remaining on the filter was determined in a liquid scintillation counter. The nonspecific binding was determined in the presence of 10 μM ryanodine.

Planar Lipid Bilayer Methods. Planar bilayers were composed of brain phosphatidylethanolamine and brain phophatidylserine (Avanti Polar Lipids, Birmingham, AL) (1:1) dissolved in decane (20 mg/mL). Purified RyRs were added to the cis chamber and fused on lipid bilayers, which were formed in a 0.2 mm diameter hole in a Lexan polycarbonate partition. In the present experiments the cis chamber is defined as the side to which RyRs are added and the opposite side is referred to as the trans chamber. In those experiments, passing currents were applied to the cis chamber and the voltage was defined with respect to the trans chamber held at ground.

The composition of the cis solution was 500 mM KCl, 1 mM EGTA, 10 mM HEPES, and 5 μM free Ca^{2+} concentration, calculated using the program by Fabiato (*35*); the pH was 7.4, adjusted with Tris-OH. The trans solution was composed of 50 mM KCl, 10 mM HEPES, and 1 mM EGTA. Triadin (4 μg/mL) was added to the cis chamber.

The channel currents were recorded at room temperature (22 ± 1 °C), amplified by a patch clamp amplifier (Axopatch 1D and pCLAMP 6, Axon Instruments Inc., Foster City, CA), and stored on a hard disk and a videocassette tape recorder through a PCM converter system (RP-880, NF Instruments, Yokohama, Japan) digitized at 28.8 kHz. Data were reproduced and low-pass filtered at 10 kHz by a filter with Bessel characteristics (octave attenuation, 48 dB), sampled at 20 kHz, and analyzed off-line on a computer (P5-200, Gateway 2000 Inc., North Sioux, ND).

RESULTS

Purification of Triadin. Two hypotheses have been presented concerning the membrane topology for triadin, based on its amino acid sequence deduced from cDNA (*25*, *36*). In both hypotheses, amino acid residues 1−47 at the N terminus have been proposed to be at least cytoplasmic.

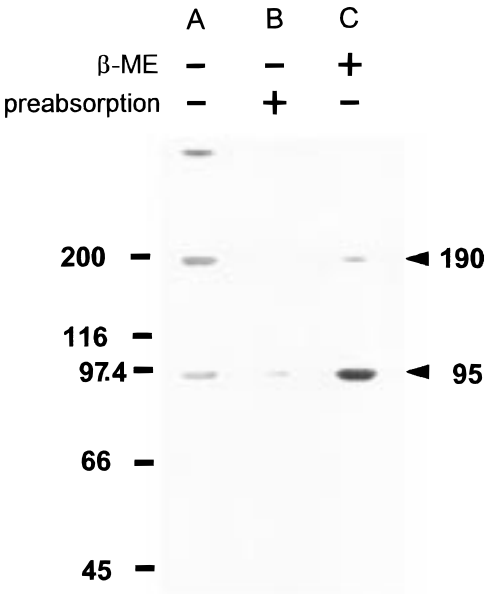

FIGURE 1: Immunostaining of the HSR with a polyclonal antibody raised against a synthetic peptide of triadin. Detailed methods are described under Materials and Methods. The HSR was treated in the presence (lane C) or absence (lanes A and B) of β-mercaptoethanol (1 M) and separated by an acrylamide gel (7.5%). In lane B, antibody preabsorbed with the conjugate of synthetic peptide of triadin and thyroglobulin was used. The arrowheads indicate the positions of monomeric (95 kDa) and dimeric (190 kDa) structures of triadin, respectively.

To analyze the regulatory mechanisms of the RyR by triadin, a polyclonal antibody was raised against a synthetic peptide of triadin corresponding to amino acid residues 33−46 at the N terminus. In the present experiment, immunostaining of the HSR with the peptide-directed antibody revealed that triadin was specifically recognized by the antibody (Figure 1; lane A). On the other hand, triadin was not recognized by the antibody preabsorbed with the synthetic peptide of triadin (lane B). Triadin was reported to be sensitive to sulfhydryl-reducing agent (*23*). When analyzed under sulfhydryl-reducing conditions, the apparent molecular mass of the oligomeric structure of triadin shifted from 190 kDa or more to 95 kDa (lane C). To examine the direct effect of triadin on the function of the RyR, triadin was purified by using the anti-triadin IgG-linked column. Purified triadin formed a large aggregate in the absence of reduction and it dissociated into 190 (dimer) and 95 kDa (monomer) bands on SDS−PAGE by the addition of sulfhydryl-reducing agent (Figure 2).

[^{3}H]Ryanodine Binding Assay. The solubilized HSR was applied to the column-linked anti-triadin IgG or preimmune IgG, and the obtained flowthrough was tested for [^{3}H]-ryanodine binding activity (Figure 3). Triadin was effectively trapped by the column-linked anti-triadin IgG but not by the column-linked preimmune IgG (data not shown). [^{3}H]Ryanodine binding activity was significantly higher in the triadin-depleted solubilized HSR, which was obtained as a flowthrough of the anti-triadin IgG-linked column. Therefore, we predicted a possibility of triadin as a negative regulator of the RyR for the first time. The effect of purified triadin on [^{3}H]ryanodine binding was investigated (Figure 4). It was found that triadin did not affect [^{3}H]ryanodine binding to the native HSR but inhibited binding to the solubilized HSR in a concentration-dependent manner. This

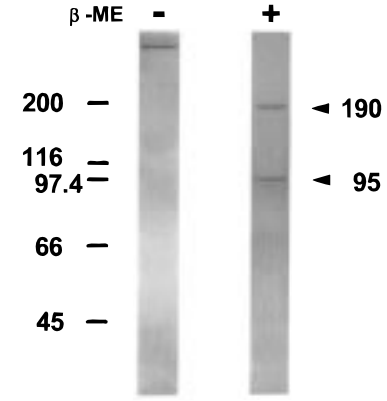

FIGURE 2: Silver staining of triadin purified with the anti-triadin IgG-linked column. Purified triadin was treated in the presence or absence of β-mercaptoethanol (1 M) and separated by an acrylamide gel (7.5%) as described in Figure 1. The arrowheads indicate the positions of monomeric (95 kDa) and dimeric (190 kDa) structures of triadin, respectively.

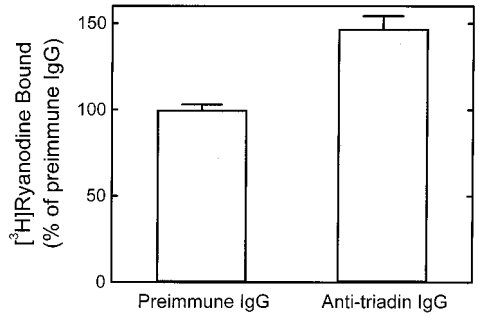

FIGURE 3: [^{3}H]Ryanodine binding to the nonadsorbed fraction of the solubilized HSR to the immunoaffinity column. Triadin-retained solubilized HSR and triadin-depleted solubilized HSR were obtained as flowthrough fractions from the column linked to preimmune IgG or anti-triadin IgG, respectively. Both samples (100 μg/mL) were incubated with [^{3}H]ryanodine (10 nM) at 37 °C in a binding buffer (0.3 M sucrose, 1 M KCl, 0.1 mM free Ca^{2+}, 20 mM Tris-HCl (pH 7.4), 0.1% soybean lecithin, and 0.1 mM *p*-APMSF) for 2 h. Poly(ethylene glycol) (1%) was added at the incubation time of 90 min. [^{3}H]Ryanodine binding was measured by the filtration method as described under Materials and Methods. Data are the means ± SE ($n = 3$) of control for the preimmune IgG.

inhibitory effect of triadin was more significantly observed on the triadin-depleted solubilized HSR. Furthermore, heat-denatured triadin did not affect the [^{3}H]ryanodine binding to the triadin-depleted solubilized HSR.

Recently, calsequestrin is reported to potentiate Ca^{2+} release through the RyR (*18*−*21*, *38*). In the present study, we also found that calsequestrin enhanced [^{3}H]ryanodine binding to the solubilized HSR (Figure 5). This stimulatory effect of calsequestrin was more eminent on the triadin-depleted solubilized HSR. Furthermore, calsequestrin did not affect [^{3}H]ryanodine binding to the native HSR, as observed with the use of triadin instead of calsequestrin, probably suggesting that calsequestrin causes effects not from the cytoplasmic side but from the luminal side of SR. It is well-known that the activities of the RyR, including Ca^{2+} release and [^{3}H]ryanodine binding, are modulated by Ca^{2+}. Ca^{2+} dependency of [^{3}H]ryanodine binding to the solubilized HSR was reduced by triadin but enhanced by calsequestrin (Figure 6). The maximum binding of [^{3}H]ryanodine was not affected by neither triadin nor calsequestrin. Furthermore, reduction of triadin by the treatment with β-mercap-

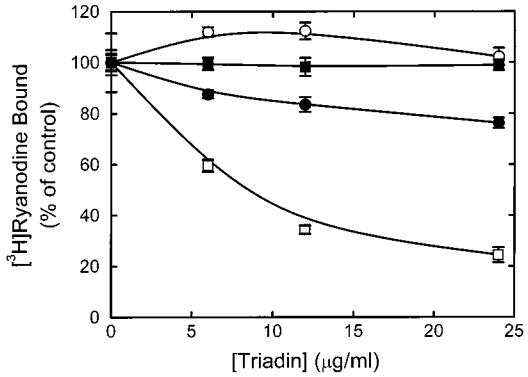

FIGURE 4: Effect of purified triadin on [^{3}H]ryanodine binding. Samples (100 μg/mL) were incubated with [^{3}H]ryanodine (10 nM) at various concentrations of native triadin (○, ●, □) or heat-denatured triadin (■). [^{3}H]Ryanodine binding was measured as described in Figure 3. (○) Native HSR; (●) the solubilized HSR; (□, ■) triadin-depleted solubilized HSR. Data are the means ± SE (n = 3) of control for each sample.

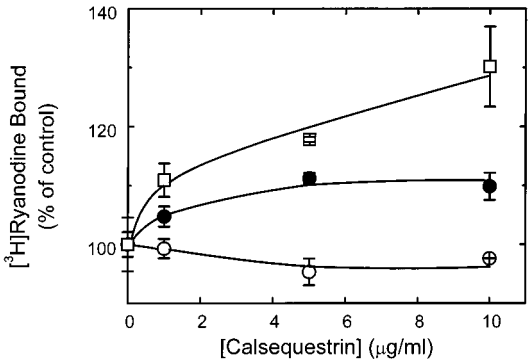

FIGURE 5: Effect of purified calsequestrin on [^{3}H]ryanodine binding. Samples (100 μg/mL) were incubated with [^{3}H]ryanodine (10 nM) at various concentrations of calsequestrin. [^{3}H]Ryanodine binding was measured as described in Figure 3. (○) Native HSR; (●) the solubilized HSR; (□) the triadin-depleted solubilized HSR. Data are the means ± SE (n = 3) of control for each sample.

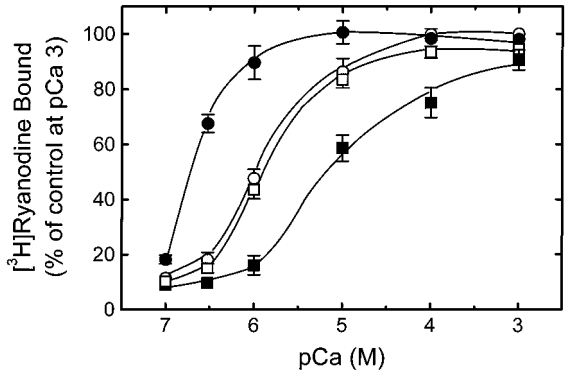

FIGURE 6: Ca^{2+} dependency of [^{3}H]ryanodine binding to the solubilized HSR. [^{3}H]Ryanodine binding to the solubilized HSR at various concentrations of Ca^{2+} were measured in the absence or presence of calsequestrin and triadin as described in Figure 3. (○) Control; (●) calsequestrin (10 μg/mL); (■) nonreduced triadin (12 μg/mL); (□) reduced triadin (12 μg/mL). Data are the means ± SE (n = 3).

toethanol abolished its inhibitory effect on the [^{3}H]ryanodine binding. To investigate whether the effects of triadin and calsequestrin on [^{3}H]ryanodine binding to the solubilized HSR were associated or not, both proteins were used in combination. The stimulatory effect of calsequestrin on [^{3}H]-

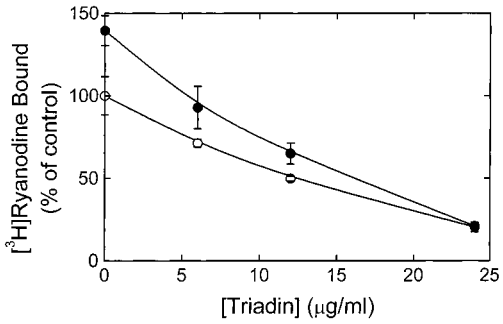

FIGURE 7: Antagonistic effect of triadin on [^{3}H]ryanodine binding to the triadin-depleted solubilized HSR enhanced by calsequestrin. [^{3}H]Ryanodine binding to the triadin-depleted solubilized HSR in the absence (○) or presence (●) of calsequestrin (10 μg/mL) was measured at various concentrations of triadin as described in Figure 3. Data are the means ± SE (n = 3) of control.

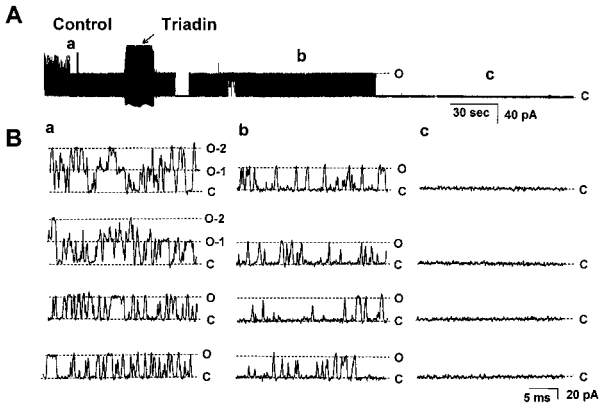

FIGURE 8: Single-channel recordings of RyR incorporated into planer lipid bilayers. (A) Continuous recordings of RyR activities before and after application of triadin (4 μg/mL). Membrane potential was held at 20 mV. Upward direction indicates current flow from cis to trans. c indicates channel close. (B) Each tracing indicated by a, b, and c in panel A is expanded in panel B. (a) Control, simultaneously openings of two channels were observed. (b) After application of triadin, channel openings were decreased. (c) RyR were completely blocked.

ryanodine binding was antagonized by triadin as shown in Figure 7.

Single-Channel Recordings of Ryanodine Receptor Ca^{2+} Release Channel. Figure 8 shows the continuous recordings of channel activities of purified RyR incorporated into the planar lipid bilayers. In the presence of 5 μM concentration of Ca^{2+} in cis solution, the openings of RyR were stably evoked. Ryanodine (5 μM) and Mg^{2+} (5 mM) when added to the cis chamber but not to the trans chamber inhibited this channel, indicating that the cis chamber is the cytoplasmic side of the SR membrane and the trans chamber is the luminal side of it. Upon application of triadin (4 μg/mL) into the cis chamber, openings of RyR were decreased and completely blocked within 3 min (n = 5). Neither application of purified triadin into the trans chamber nor that of heat-denatured triadin into the cis chamber caused a change in the RyR activity (data not shown) (n = 4).

Analysis of the Interaction between Triadin and Calsequestrin. Although the stimulatory effect of calsequestrin on [^{3}H]ryanodine binding was abolished by triadin, it is possible that triadin and calsequestrin affect the binding in independent ways, without any interaction between the two proteins. To reveal the possibility of such interaction, we

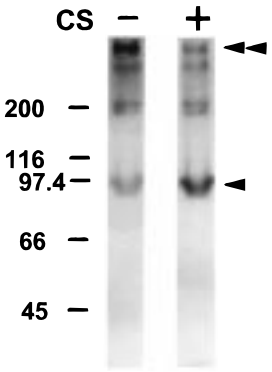

FIGURE 9: Inhibitory effect of calsequestrin on the formation of oligomeric structure of triadin. Immunostaining of the calsequestrin-depleted JFM with anti-triadin antibody was performed as described under Materials and Methods. Samples were treated in the presence or absence of calsequestrin (40 μg/mL) and separated by an acrylamide gel (7.5%). Double and single arrowheads indicate oligomeric and monomeric triadins, respectively.

investigated the effect of calsequestrin on the oligomeric structure of triadin. After incubation of calsequestrin-depleted JFM with or without calsequestrin, triadin was detected by Western blotting. As shown in Figure 9, triadin oligomer (double arrowhead) dissociated into a monomer (single arrowhead) in the presence of calsequestrin, suggesting the presence of the interaction between triadin and calsequestrin.

DISCUSSION

In skeletal muscle, triadin is specifically enriched in the junctional SR, where it colocalizes with the RyR, forming oligomer on the SR membrane through disulfide bonds (*23*−*25*). Triadin has been proposed to function as a signal transducer between the dihydropyridine receptor and RyR (*23*−*28*). Recently, triadin has been shown to interact with RyR and calsequestrin in the lumen of the SR (*29*). Thus, it is likely that the RyR is regulated by receiving signals not only from the dihydropyridine receptor but also from calsequestrin perceiving the luminal Ca^{2+} concentration in the SR. Here, we first tested the direct effects of purified triadin and calsequestrin on RyR function.

Triadin was purified from the solubilized HSR by an anti-triadin immunoaffinity column, and at the same time, the triadin-depleted solubilized HSR was obtained as a flow through of the immunoaffinity column. On the other hand, triadin-retained solubilized HSR was obtained as a flow through of the column-linked preimmune IgG, instead of anti-triadin IgG. [^{3}H]Ryanodine binding activity was significantly higher in the triadin-depleted solubilized HSR than in the triadin-retained one. Therefore, we tested the possibility that triadin was a negative regulator of the RyR. Purified triadin was demonstrated to inhibit the [^{3}H]ryanodine binding to the solubilized HSR, and the inhibitory effect was more eminent in the triadin-depleted solubilized HSR. It is an important finding that the openings of purified RyR were abolished by the addition of triadin. We have successfully provided the evidence that triadin functions as a negative regulator of the RyR.

Calsequestrin, a major protein in the lumen of the SR for Ca^{2+} binding with moderate affinity and high capacity (*16*) is anchored to the luminal face of the junctional SR and is thought to sequester and concentrate Ca^{2+} near the RyR (*17*). Recently, another important role of calsequestrin has attracted attention; namely, adding calsequestrin to the luminal side of the SR evoked potentiation of Ca^{2+} release and channel current mediated through the RyR (*18*−*21*, *38*). Along with these reports, myotoxin *a*, a novel type of Ca^{2+}-releasing agent that does not bind to the RyR, was found to work only in the presence of calsequestrin (*21*, *22*). The present paper revealed that calsequestrin enhanced [^{3}H]ryanodine binding to the solubilized HSR, as predicted from the previous studies.

[^{3}H]Ryanodine binding was demonstrated to be enhanced by calsequestrin but reduced by triadin. To investigate whether these opposite effects of triadin and calsequestrin were related or not, both proteins were used singly or in combination and their effects on [^{3}H]ryanodine binding to the solubilized HSR were analyzed. Expectedly, the effect of calsequestrin on [^{3}H]ryanodine binding was antagonized by triadin. Further witness to the interaction between triadin and calsequestrin was the fact that adding calsequestrin inhibited the formation of the oligomeric structure of triadin, proved by immunostaining of the calsequestrin-depleted JFM with anti-triadin antibody in the presence or absence of calsequestrin. Inhibitory action of triadin on [^{3}H]ryanodine binding was abolished by the reduction of triadin, which caused a shift from its oligomeric structure to a monomeric one. It is consistent with the observation that sulfhydryl-reducing agent caused contraction of skeletal muscle through triggering Ca^{2+} efflux from the SR (*39*). A possible explanation for these observations is that calsequestrin regulates the RyR via the conformational change of triadin as a inhibitory factor.

It has been reported that triadin associates with both the RyR and calsequestrin in the lumen of the SR in a Ca^{2+}-dependent fashion and its amino acid sequence 100−706 may be important for the binding to the RyR and calsequestrin (*29*). However, Fan et al. (*36*) have proposed a model in which the cytoplasmic region of triadin (amino acid residues 110−163) interacts with RyR at the cytoplasmic side. The addition of triadin to the cis chamber (cytoplasmic side) significantly reduced the gating activity of RyR incorporated into the planar lipid bilayer, but no remarkable effect was observed upon the addition of triadin to the trans chamber (luminal side). Therefore, our data support the model proposed by Fan et al. (*36*) in which the interaction between the RyR and triadin can occur at the cytoplasmic side of SR.

A remaining issue in our hypothesis is how the interaction between triadin and luminally trapped calsequestrin can be achieved in the SR membrane and the role of luminal Ca^{2+} in its mechanism. Calsequestrin can associate with triadin at the luminal side in a Ca^{2+}-dependent fashion (*29*). The addition of triadin to the cytoplasmic side of native HSR reduced the [^{3}H]ryanodine binding only in the presence of A23187, a representable Ca^{2+} ionophore (Fujimori et al., unpublished observation). Although these findings suggest the important role of luminal Ca^{2+} in the regulatory mechanism of Ca^{2+} release through RyR, further studies are necessary, including the identification of sites on triadin and calsequestrin that affect the RyR function and analysis of their interaction with the RyR and their dependency on luminal Ca^{2+}.

In summary, triadin is demonstrated to play an important role in the inhibitory regulatory mechanism of the Ca^{2+} release through RyR. Our present data provide the first direct evidence that the skeletal muscle RyR is regulated negatively by triadin and positively by calsequestrin, whose steps may involve their interaction.

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