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Functional Interaction of the Cytoplasmic Domain of Triadin with the Skeletal Ryanodine Receptor*

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Triadin has been shown to co-localize with the ryanodine receptor in the sarcoplasmic reticulum membrane. We show that immunoprecipitation of solubilized sarcoplasmic reticulum membrane with antibodies directed against triadin or ryanodine receptor, leads to the co-immunoprecipitation of ryanodine receptor and triadin. We then investigated the functional importance of the cytoplasmic domain of triadin (residues 1–47) in the control of Ca^{2+} release from sarcoplasmic reticulum. We show that antibodies directed against a synthetic peptide encompassing residues 2–17, induce a decrease in the rate of Ca^{2+} release from sarcoplasmic reticulum vesicles as well as a decrease in the open probability of the ryanodine receptor Ca^{2+} channel incorporated in lipid bilayers. Using surface plasmon resonance spectroscopy, we defined a discrete domain (residues 18–46) of the cytoplasmic part of triadin interacting with the purified ryanodine receptor. This interaction is optimal at low Ca^{2+} concentration (up to *p*Ca 5) and inhibited by increasing calcium concentration (IC_{50} of 300 μM). The direct molecular interaction of this triadin domain with the ryanodine receptor was confirmed by overlay assay and shown to induce the inhibition of the Ca^{2+} channel activity of purified RyR in bilayer. We propose that this interaction plays a critical role in the control, by triadin, of the Ca^{2+} channel behavior of the ryanodine receptor and therefore may represent an important step in the regulation process of excitation-contraction coupling in skeletal muscle.

In skeletal muscle cell, plasma membrane depolarization leads to the release of calcium from the sarcoplasmic reticulum (SR)[1] (1). This process, named excitation-contraction coupling, takes place in a specific region of the cell where SR membrane faces the plasma membrane to form the triad. Two proteins are mainly involved in this process (2). The dihydropyridines receptor (DHPR) is localized in the plasma membrane and senses its depolarization (3–5); the ryanodine receptor (RyR), localized in the SR membrane, represents the exit way for Ca^{2+} of the SR (6–8) (for review see Ref. 9). *In vivo*, RyR opening is thought to be triggered by a charge movement induced conformational change of the DHPR (10, 11). This "mechanical coupling" hypothesis is supported by a number of results showing the existence of a complex involving both RyR and DHPR (12–14). *In vitro*, Ca^{2+} release from SR has been shown to be modulated by a number of effectors such as ATP, Mg^{2+}, caffeine, ryanodine, and Ca^{2+} itself (8, 15–17).

Moreover, during recent years an increasing number of proteins located in the triad have been shown to be able to regulate Ca^{2+} release from SR. Some of these proteins, such as the FK506-binding protein (FKBP12) and the cytoplasmic Ca^{2+}-binding proteins calmodulin and S100A1, interact directly with the RyR and modulate its Ca^{2+} channel behavior (18–22). A different set of results indicates that Ca^{2+} efflux through the RyR Ca^{2+} channel is also controlled by luminal Ca^{2+} concentration presumably via a functional interaction between RyR and calsequestrin, the major Ca^{2+}-binding protein of the SR lumen (23, 24).

Different groups have proposed that another protein named triadin is involved in the regulation of Ca^{2+} release from SR. Triadin is of particular interest because it is one of the SR membrane proteins that co-localize with RyR (26, 27). Skeletal triadin is a 95-kDa glycoprotein (26, 28). Based on its primary sequence analysis and accessibility to specific antibodies and protease, a model has been proposed for the membrane topography of skeletal triadin in the SR membrane (28, 29). This model shows that triadin is composed of a small cytoplasmic region (amino acid 1–47), followed by a single transmembrane domain and a large luminal region. Although different results have suggested an interaction of triadin with either RyR, DHPR , or calsequestrin (30–33), the exact role of triadin in the control of the excitation-contraction coupling mechanism as well as the exact domains of triadin involved in an interaction with RyR, have still to be determined.

In this study, we investigated the functional interaction between the cytoplasmic domain of triadin and the RyR. We show that antibodies specifically directed against the N-terminal cytoplasmic extremity of triadin inhibit caffeine-induced Ca^{2+} release from SR vesicles by reducing the open probability of the RyR Ca^{2+} channel. Surface plasmon resonance measurements revealed a specific interaction of the cytoplasmic portion of triadin with purified RyR. This was confirmed by overlay assays, which showed this interaction to be direct. This interaction is dependent on the free Ca^{2+} concentration and is completely inhibited in the presence of several millimolar of $CaCl_2$. These results represent the first identification of a discrete domain of triadin directly involved in a functional interaction with RyR. This interaction would allow a direct control of RyR

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; HSR, heavy SR; RyR, ryanodine receptor; DHPR, dihydropyridines receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; Ab, antibody.

Ca^{2+} channel behavior by triadin from the cytosolic side and therefore strongly suggests that triadin is a major actor of excitation-contraction coupling *in vivo*.

EXPERIMENTAL PROCEDURES

Membrane Preparation—Heavy sarcoplasmic reticulum (HSR) vesicles were prepared from rabbit skeletal muscle as described previously (14).

RyR Purification—Ryanodine receptor was purified on sucrose gradient after solubilization of HSR membrane with CHAPS as described by Lai *et al.* (8). The fractions corresponding to the peak of bound [^{3}H]ryanodine were pooled and concentrated by filtration on a YM30 membrane (Amicon Corp).

Synthetic Peptides—Two peptides corresponding to residues 2–17 (peptide 1) and 18–46 (peptide 2) of the skeletal triadin (28) were synthesized with an exogenous C-terminal Tyr and Cys, respectively. A third peptide (peptide 3), not related to triadin, was used for the surface plasmon resonance control experiments of RyR-peptide 2 interaction. This peptide was chosen because of its isoelectric point (pI 9.13) and molecular mass (2035 Da), which were similar to those of peptide 2 (pI 8.98, molecular mass 3180,9 Da). For overlay assays, peptide 2 was synthesized with an exogenous C-terminal biotinylated Lys.

Antipeptides Antibodies—Antibodies directed against synthetic peptides corresponding to amino acids 2–17 (A-Nter) or 691–706 (A-Cter) extremity of triadin were obtained and characterized as described previously (29). These antibodies were affinity purified against the corresponding peptide. Fab fragments of affinity purified A-Nter antibodies were prepared using a Fab preparation kit (Pierce).

Immunoprecipitation—Heavy SR vesicles were solubilized in the presence of CHAPS as described in Ref. 14. Solubilized proteins were then incubated with antibodies specifically directed against ryanodine receptor (anti-RyR) or either N-terminal (A-Nter) or C-terminal (A-Cter) extremity of triadin. The immune complex was then precipitated with protein A-Sepharose, and the immunoprecipitated proteins identified by Western blot analysis using a chemiluminescent technique after electrophoretic separation and transferred to an Immobilon sheet. A-Nter and A-Cter antibodies as well as anti-RyR antibodies used in these experiments were previously described by Marty *et al.* (29).

Ca^{2+} Flux Measurements—HSR vesicles (10 mg/ml) were incubated for 14 h in ice in the presence or in the absence of A-Nter antibodies (HSR protein/Ab ratio of 10 or 20). For control experiments, A-Nter antibodies were preincubated with an excess of corresponding peptide before their incubation with HSR vesicles. Ca^{2+} uptake and Ca^{2+} release were then monitored using arsenazo III as a Ca^{2+} indicator as described previously (16). The arsenazo III signal was converted to nanomoles of Ca^{2+} by determining the Δ arsenazo III signal/Δ [Ca^{2+}] coefficient from a calibration curve. The concentration of the contaminant Ca^{2+} brought by HSR vesicles and buffer was calculated to be 20 μM.

For active Ca^{2+} loading of HSR vesicles, the vesicles (0.25 mg/ml) were incubated in a solution containing, 0.15 M KCl, 5.0 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 2 mM $MgCl_2$, 12.5 μM $CaCl_2$, 34 μM arsenazo III and 20 mM MES, pH 6.8 (solution A). After incubation for 15 min at room temperature, Ca^{2+} uptake was induced by addition of 1 mM ATP.

For Ca^{2+} release experiments, HSR vesicles were actively loaded as described above, and then mixed with 1 volume of solution B containing 0.15 M KCl, 34 μM arsenazo III, 10 mM caffeine, and 20 mM MES, pH 6.8. The time course of HSR Ca^{2+}-release was monitored using a stopped flow apparatus (Bio-Logic SFM-3). Ten to fifteen traces, each representing 4,000 data points of the arsenazo III signal, were averaged for each experiment.

RyR Ca^{2+} Channel Reconstitution and Single Channel Recording Analysis—Lipids bilayers were cast from a phospholipid solution in *n*-decane containing a 5:2:3 mixture of phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine (30 mg/ml). The voltage control side was the *cis* chamber, and the *trans* chamber was referred to as ground. HSR membrane vesicles or purified RyR were applied on top of the preformed bilayer from the *cis* side. The experimental solutions were as follows: for the *cis* chamber, 10 mM HEPES, pH 7.0, 250 mM NaCl, 1 mM ATP, 0.1 mM $CaCl_2$, 0.1 mM EGTA (*p*Ca 5.5); and for the *trans* chamber, 10 mM HEPES, pH 7.0, 50 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM EGTA (*p*Ca 5.5). Traces were recorded at 0 mV. Single channel recording and analysis were performed as described previously (21).

Real Time Surface Plasmon Resonance Recording—Real time surface plasmon resonance experiments were performed on a BIAcore biosensor system (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were performed at 25 °C with a constant flow rate of 10 μl/min. Synthetic peptides were directly coupled to a carboxymethylated dextran matrix (CM5 sensor chip, Pharmacia Biosensor). Peptide 1 was coupled through its amino groups according to the protocol provided by Pharmacia Biosensor. Peptides 2 and 3 were immobilized through their unique C-terminal thiol group according to the protocols provided by Pharmacia Biosensor and modified as follows. Before peptide injection, excess of reactive carboxyl groups was inactivated with 35 μl of 1 mM ethanolamine hydrochloride, pH 8.5. After peptide immobilization, excess of reactive disulfide groups were neutralized with 25 μl of 1 M NaCl, 50 mM cysteine in 0.1 M formate, pH 4.3, and noncovalently bound peptides removed by a wash with 20 μl of 50 mM HCl. The concentration of peptide used was adjusted to obtain an equivalent level of immobilization (expressed in fmol/mm^2) calculated according to the ratio of 1,000 units/ng of peptide immobilized/mm^2 given by Pharmacia Biosensor. Purified RyR, previously dialyzed overnight at 4 °C against a buffer containing 10 mM HEPES, pH 7.4, and 150 mM NaCl was injected in the same buffer containing different concentrations of EGTA and $CaCl_2$ to obtain the wanted values of *p*Ca.

Overlay Assays—2 μg of purified skeletal RyR were separated on 5–15% SDS-polyacrylamide gel electrophoresis and transferred on an Immobilon sheet. The Immobilon sheet was then blocked with 1% bovine serum albumin for 30 min at room temperature in buffer A (150 mM NaCl, 1 mM EGTA, 0.1% T20, 10 mM HEPES, pH 7.4). Overlay was carried out with 50 nM of biotinylated peptide 2 for 2 h at room temperature in presence of 1% bovine serum albumin in buffer A. At the end of the incubation, the blot was washed 3 times for 5 min in buffer A. Peptide 2 binding proteins were revealed by chemiluminescent technique using peroxidase-conjugated anti-biotin monoclonal antibodies (Jackson ImmunoResearch Laboratories, Inc.).

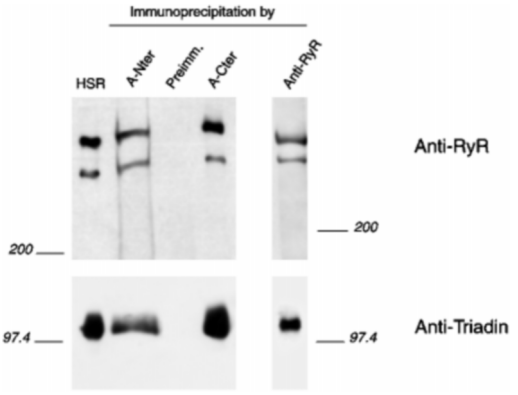

FIG. 1. **Immunoprecipitation of solubilized HSR proteins with antibodies directed against triadin or ryanodine receptor.** Solubilized HSR proteins were immunoprecipitated as described under "Experimental Procedures" with A-Nter antibodies, preimmune serum, A-Cter antibodies, or anti-RyR antibodies. The immune complexes formed were analyzed in Western blot with anti-RyR or anti-triadin (A-Cter) antibodies. The first lane (*HSR*) corresponds to Western blot analysis of HSR proteins revealed by anti-RyR antibodies or anti-triadin antibodies.

RESULTS AND DISCUSSION

Immunoprecipitation of SR Proteins Using Antibodies Directed Against Triadin or RyR—The interaction between ryanodine receptor and triadin was first tested by co-immunoprecipitation experiments with specific antibodies. Solubilized HSR proteins were immunoprecipitated with antibodies directed against N-terminal (A-Nter) or C-terminal (A-Cter) domain of triadin or with antibodies directed against RyR, as described under "Experimental Procedures." Immunoprecipitated proteins were then analyzed by Western blot (Fig. 1) with antibodies directed against either RyR or triadin. Fig. 1 shows that immunoprecipitation by A-Nter or A-Cter leads to the co-precipitation of RyR and triadin, as indicated by the labeling of the immune complex by both anti-RyR and A-Cter antibodies, whereas preimmune serum is unable to precipitate either of this two proteins. Conversely, immunoprecipitation with antibodies directed against RyR leads to the co-precipitation of

RyR and triadin as indicated by immunobloting of the precipitated proteins with A-Cter and anti-RyR antibodies. These results confirm the existence of a complex containing both RyR and triadin (30, 31, 39).

Effect of Anti-triadin Antibodies on the Functional Properties of the RyR Ca^{2+} Channel—To investigate the functional role of the RyR-triadin interaction, we tested the effect of anti-triadin antibodies on Ca^{2+} uptake and Ca^{2+} release from SR vesicles as well as on the RyR Ca^{2+} channel behavior. Using proteolytic degradation techniques, we have previously shown that the first 47 amino acids of triadin are protruding into the cytoplasm, whereas the rest of the protein is localized within the SR lumen (29). We therefore focused our study on the role of the cytoplasmic domain of triadin in the regulation of Ca^{2+} release from SR and tested the effect of the A-Nter antibodies, directed against residues 2–17 of triadin, on Ca^{2+} release from SR vesicles. SR vesicles, incubated in the presence or absence of A-Nter antibodies, were actively loaded with Ca^{2+} and then tested for Ca^{2+} release, as described under "Experimental Procedures." As shown in Fig. 2*A*, representing the time course of Ca^{2+} uptake by SR vesicles preincubated in the absence (*trace A*) or presence (*trace B*) of A-Nter antibodies, these antibodies produce no significant effect on the active Ca^{2+} loading of the SR vesicles. Under the conditions used, the loading level was 103.4 ± 14.0 (n = 6) and 97.3 ± 5.8 (n = 8) nmol/mg for SR vesicles incubated in the absence or presence of A-Nter, respectively. In contrast, under the same conditions, incubation of the SR vesicles with A-Nter antibodies induces a clear inhibition of the Ca^{2+} release measured in the presence of caffeine (Fig. 2*B, curves B* and *C* compared with *curve A*). The initial rate of Ca^{2+} release, calculated from the slope of Ca^{2+} release curve at time 0, decreased from 288 nmol of Ca^{2+} mg^{-1} s^{-1} protein, *trace A* (no antibodies), to 96 and 37 nmol of Ca^{2+} mg^{-1} s^{-1} protein, *trace B* (proteins/A-Nter = 20) and *trace C* (proteins/A-Nter = 10). No significant change of Ca^{2+} release rate was observed when SR vesicles were incubated with the peptide alone (data not shown) or with A-Nter antibodies pre-incubated with the corresponding peptide (*curve D*). These results clearly demonstrate that antibodies directed against the cytoplasmic domain of triadin inhibit Ca^{2+} release from SR vesicles. Therefore, modifying triadin can induce a modification of RyR function. However this effect of A-Nter antibodies could result from modification by the antibodies of either the RyR Ca^{2+} channel properties, or any other step of the Ca^{2+} release process.

We then studied the effect of A-Nter antibodies on the intrinsic properties of the RyR Ca^{2+} channel after incorporation of HSR vesicles in planar lipid bilayer. Fig. 3 shows the single channel recording and current integral obtained using Na^+ as the current carrier. In control conditions (pCa 5.5 and 1 mM ATP), we observed a high conductance channel (490 pS) (Fig. 3, *left trace*). In the *cis* chamber, the addition of Fab fragment of the A-Nter induces a decrease of the normalized open probability of the channel from 0.25 (control conditions) to 0.03 (*right trace*), corresponding to an inhibition of 73 ± 8% (n = 4) of the RyR current. Neither addition of antigenic peptide alone nor that of A-Nter antibodies preincubated with the peptide induces a change of Ca^{2+} channel activity (data not shown). This channel was previously identified as the RyR Ca^{2+} channel based on its sensitivity to Ca^{2+}, ryanodine, and ruthenium red (21). When purified RyR was incorporated into lipid bilayers, no effect of the A-Nter triadin antibodies on RyR Ca^{2+} channel activity was observed (data not shown), confirming the fact that the effect of A-Nter antibodies on RyR must occur via triadin. These data strongly suggest that the inhibition of the Ca^{2+} release from SR by A-Nter antibodies results from the reduction of the RyR Ca^{2+} channel open probability by these antibodies.

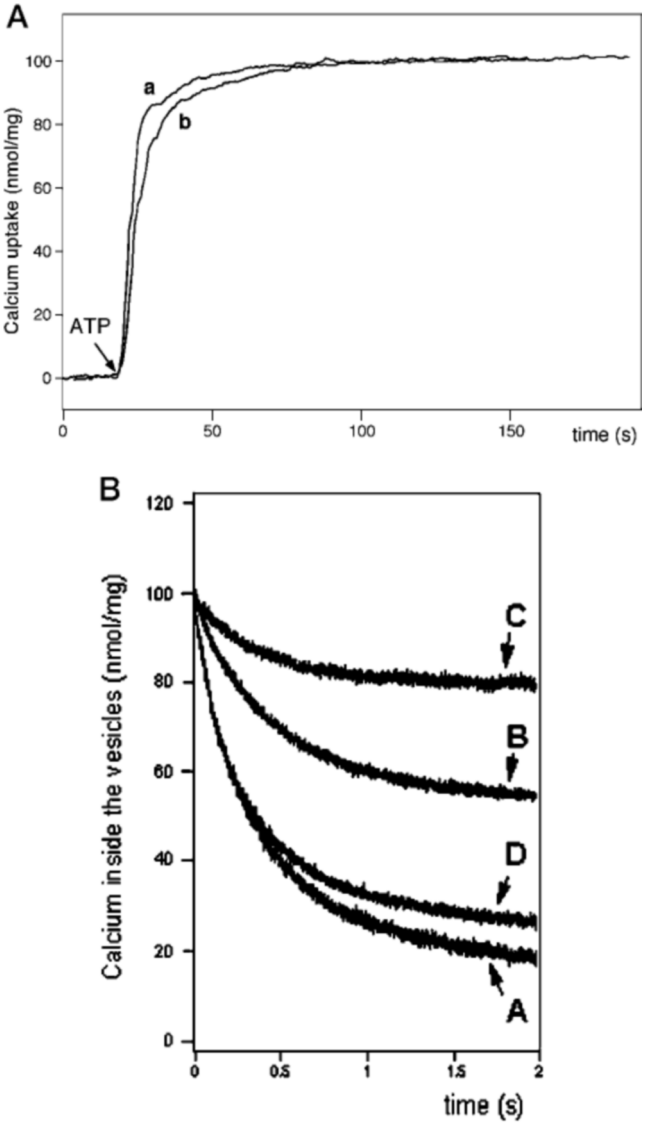

FIG. 2. **Effect of A-Nter antibodies on Ca^{2+} uptake (*A*) and Ca^{2+} release (*B*) from SR vesicles.** *A*, Ca^{2+} uptake. HSR vesicles incubated in the absence of antibodies (*curve a*) or presence of A-Nter antibodies (*curve b*) (protein/Ab = 10) were actively loaded in the presence of ATP-Mg. Each curve represents the average of four different experiments. The amount of Ca^{2+} loaded, determined as described under "Experimental Procedures" was 103.4 ± 14.0 (mean ± S.D., n = 6) and 97.3 ± 5.8 (mean ± S.D., n = 8) nmol/mg of SR vesicles incubated in the absence or presence of A-Nter antibodies (protein/Ab = 10 mg/mg), respectively. *B*, time course of Ca^{2+} release. HSR vesicles preincubated in the absence of antibodies (*curve A*), in presence of A-Nter antibodies (*curves B* (protein/Ab = 20 mg/mg) and *C* (protein/Ab = 10 mg/mg)), or in the presence of A-Nter antibodies (protein/Ab = 20 mg/mg) preincubated with corresponding peptide (*curve D*) (Ab/peptide = 6 mg/mg) were actively loaded with Ca^{2+} for 15 min. Ca^{2+} release was then induced in presence of caffeine as described under "Experimental Procedures." Data represent the average of 10 to 15 traces (each representing 4,000 data points) and are representative of at least three experiments.

Molecular Interaction of RyR with the N-terminal Domain of Triadin—The effect of A-Nter antibodies on the RyR Ca^{2+} channel properties described above could be due to the modification of a direct interaction between RyR and the cytoplasmic N-terminal region of triadin or an at-distance modification of the interaction between another region of triadin and RyR. The existence of a direct molecular interaction between RyR and the N-terminal region of triadin was then addressed using

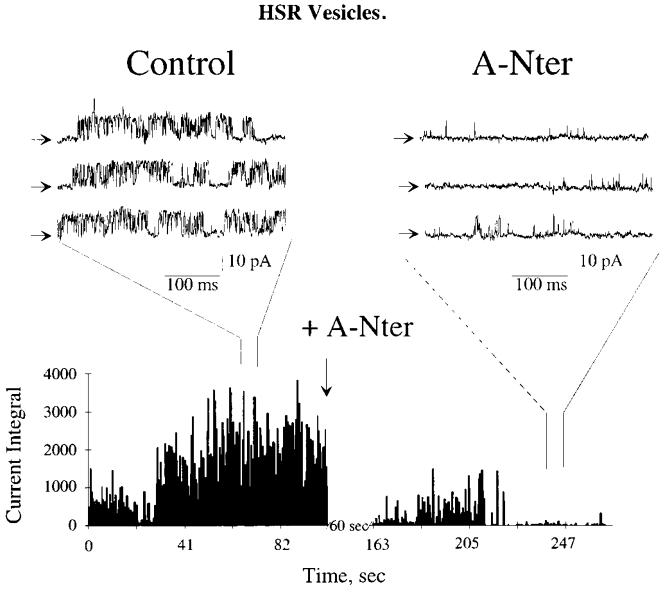

FIG. 3. **Effect of A-Nter antibodies on skeletal RyR Ca^{2+} channel incorporated into lipid bilayers.** HSR vesicles were fused with lipid bilayer and single channel recording and current integral *versus* time were measured as described under "Experimental Procedures." Current integral values are 1429 pA·ms before addition of antibodies (*control*), and 178 pA·ms after addition of Fab fragment of A-Nter antibodies (1/110). *Horizontal arrows* indicate the closed state of the channel. This experiment is representative of four experiments.

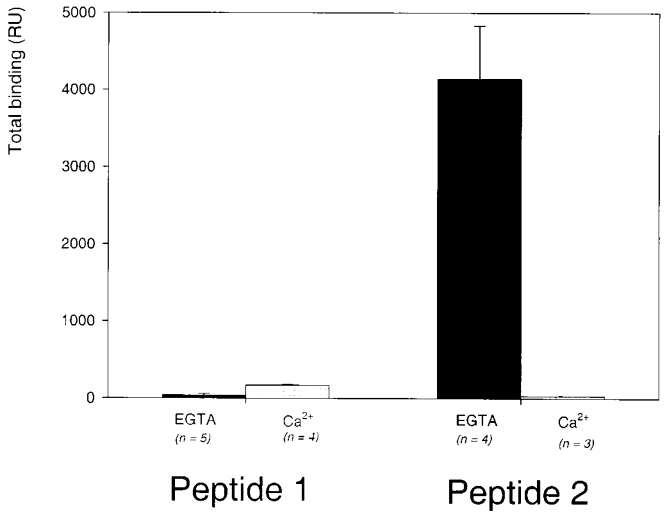

FIG. 4. **Interaction of purified RyR with peptide 1 and peptide 2.** Peptides 1 and 2 corresponding to residues 2–17 and 18–46 of cytoplasmic domain of triadin, respectively, were immobilized on the sensor chip surface as described under "Experimental Procedures." 1.4 and 1 μg of purified RyR were injected on immobilized peptides 1 (169 fmol/mm^2) and 2 (58–184 fmol/mm^2), respectively, in presence of 1 mM EGTA or 2 mM Ca^{2+} as described under "Experimental Procedures." To facilitate the comparison between the results obtained with peptides 1 and 2, RyR interaction signal was normalized for a peptide immobilization value of 169 fmol/mm^2. Data are expressed as mean ± S.D. of total purified RyR binding.

surface plasmon resonance technique. For these experiments, two peptides corresponding to residues 2–17 (peptide 1) and 18–46 (peptide 2) of triadin, respectively, were synthesized and immobilized to the dextran matrix coating the sensor chip. The interaction of each peptide with purified RyR was then monitored using the optical biosensor BIAcore. Fig. 4 depicts the amplitude of the binding signal corresponding to the interaction of purified RyR with peptides 1 and 2 in the presence of 1 mM EGTA or 2 mM Ca^{2+}. Of these two peptides, only peptide 2 interacts with purified RyR in the presence of EGTA but not in the presence of 2 mM Ca^{2+}. No interaction of purified RyR with peptide 1 was observed in the presence of EGTA or Ca^{2+}. Accessibility of immobilized peptide 1 was ensured by its reactivity with A-Nter antibodies (data not shown).

In the next set of experiments, we analyzed in more detail the interaction of purified RyR with peptide 2 in the presence of 1 mM EGTA. Fig. 5, *upper trace*, shows the sensorgrams representing the real time interaction of peptide 2 with increasing concentrations of purified RyR. Fig. 5, *lower trace* shows that the resonance unit response corresponding to the binding of purified RyR on peptide 2 is dose-dependent and approaches the saturation for concentrations of purified RyR close to 200 nM with an apparent affinity of 27 ± 5 nM (n = 4). The interaction between the purified RyR and the immobilized peptide 2 could be inhibited by preincubation of RyR with peptide 2 (data not shown). Nonspecific interaction between purified RyR and the activated surface sensor was measured on either a surface coated with peptide 3, unrelated to triadin (Fig. 5, *curve E*), or a surface sensor without immobilized peptide. In both cases, the nonspecific interaction was consistently found to represent less than 10% of the total binding signal.

However the surface plasmon resonance interaction signal described above could reflect the interaction of triadin with RyR via another protein present in the purified RyR preparation. To test this hypothesis we performed overlay of biotinylated peptide 2 on purified RyR. Fig. 6, *lane 1*, shows that immunolabeling by anti-biotin antibodies of purified RyR preparation overlaid with biotinylated peptide 2 leads to the labeling of two bands corresponding to the high molecular weight protein RyR and to a lower molecular mass peptide (approximately 58 kDa), respectively. However labeling of the 58-kDa peptide was also observed when overlay was performed in the absence of peptide 2 (Fig. 6, *lane 2*) and is therefore likely due to a nonspecific labeling by the anti-biotin antibodies themselves. These results clearly demonstrate the direct molecular interaction between RyR and peptide 2 in presence of 1 mM EGTA.

Therefore we investigated the effect of the free Ca^{2+} concentration on the interaction of RyR with peptide 2. Purified RyR, at a fixed concentration, was injected on the peptide-coated sensor surface in a buffer containing different free Ca^{2+} concentrations. Fig. 7 represents the variation of the specific binding signal amplitude as a function of [Ca^{2+}] and shows that RyR-peptide 2 interaction is optimal at low Ca^{2+} concentrations (below micromolar) and is inhibited by increasing [Ca^{2+}] with IC_{50} for Ca^{2+} in the range of 300 μM. These results clearly indicate that purified RyR interacts directly with the peptide corresponding to residues 18–46 of triadin, this interaction being regulated by Ca^{2+}.

Effect of Peptide 2 on the Functional Properties of the Purified RyR Ca^{2+} Channel—Fig. 8 shows the recording of channel activity of purified RyR incorporated into lipid bilayers. As mentioned above, addition of A-Nter triadin antibodies to purified RyR incorporated to lipid bilayer did not induce any change in the RyR activity, indicating that the effect of these antibodies on RyR occur via triadin. Therefore we tested the effect of peptide 2 on the activity of the purified RyR incorporated into lipid bilayer. In the presence of 1 mM ATP and 3 μM free Ca^{2+} in the *cis* chamber, RyR was consistently open as shown on Fig. 8 (*control*). The addition of peptide 2 (100 nM final concentration) in the *cis* chamber induces a decrease of the normalized open probability of the channel from 0.19 to 0.04 corresponding to a 79% inhibition of the current. These results demonstrate that the molecular interaction of peptide 2 with purified RyR induces the inhibition of the RyR channel activity.

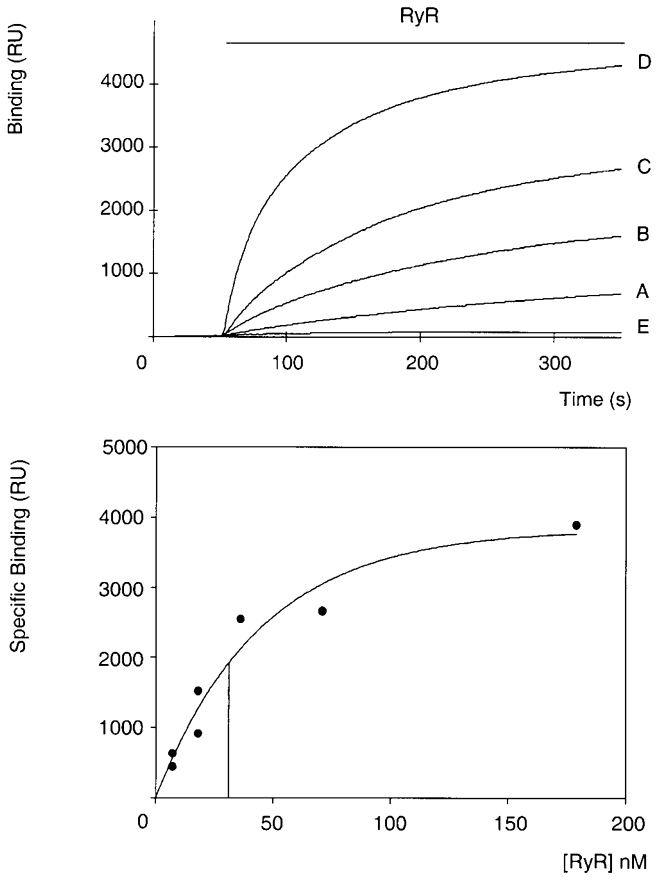

FIG. 5. **Interaction of purified RyR with peptide 2 in absence of Ca^{2+}.** Purified RyR was injected at various concentrations on the peptide 2 immobilized (142 fmol/mm^2) on the sensor chip surface (upper trace). *Curve A*, 200 ng of RyR; *curve B*, 500 ng of RyR; *curve C*, 1 μg of RyR; *curve D*, 5 μg of RyR. *Curve E* represents the binding of RyR (500 ng) with peptide 3. *Lower trace*, amplitude of the specific binding signal (total nonspecific) corresponding to the interaction of purified RyR with peptide 2 as a function of [RyR]. The *curve* represents the best fit of the data by the equation $y = a(1 - \exp(-bx))$. This experiment is representative of four different sets of experiments. The calculated apparent affinity of RyR for peptide 2 is 27 ± 5 nM (mean ± S.D., $n = 4$).

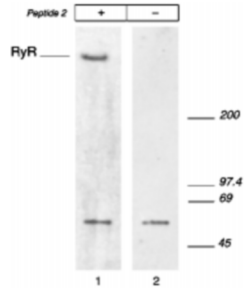

FIG. 6. **Biotinylated peptide 2 overlay of purified RyR.** 2 μg of purified RyR preparation were separated on 5–15% SDS-polyacrylamide gel electrophoresis and blotted onto Immobilon sheets as described under "Experimental Procedures." Ligand overlay was carried out for 2 h at room temperature in a buffer containing 150 mM NaCl, 1 mM EGTA, 0.1% T20, 1% bovine serum albumin, 10 mM HEPES, pH 7.4, in the presence (*lane 1*) or the absence (*lane 2*) of 50 nM biotinylated peptide 2 of triadin. Peptide 2-binding proteins were revealed with monoclonal anti-biotin antibodies.

In conclusion, several new aspects of the interaction of triadin and RyR are described here. Our results represent the first evidence of a direct interaction of the cytoplasmic domain of triadin with RyR. Moreover, these results allow us to define a discrete domain restricted to the residues 18–46 of this cytoplasmic portion of triadin as responsible for the interaction

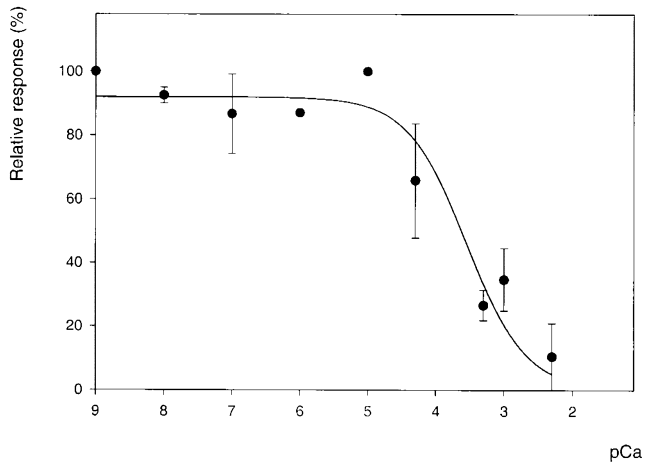

FIG. 7. **Effect of Ca^{2+} on the interaction of purified RyR with peptide 2.** Peptide 2 was immobilized on the sensor chip surface as described under "Experimental Procedures" and its interaction with purified RyR (1.5 μg) measured in a buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM EGTA, and various [Ca^{2+}] to obtain various pCa values. Specific binding values were normalized to percentage of the maximal binding value measured at pCa 9. The curve represents the best fit of the data by the equation $y = a(b/(b + e(-2.3x)))$, providing an IC_{50} value for Ca^{2+} of 300 μM.

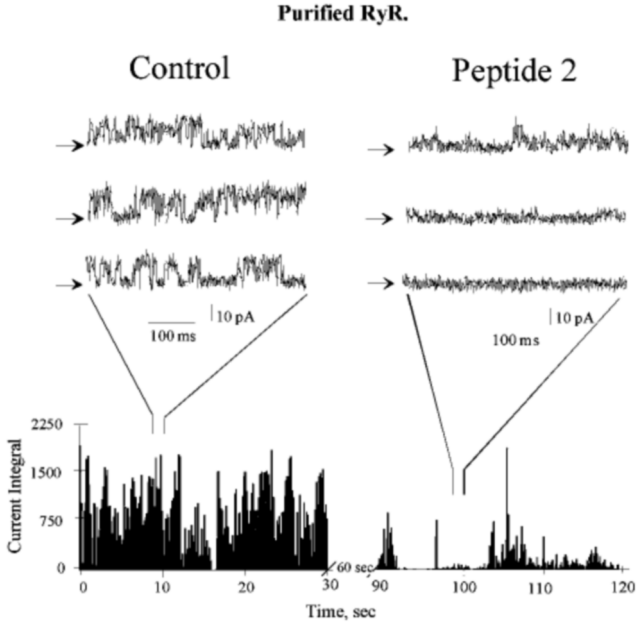

FIG. 8. **Effect of peptide 2 on purified RyR Ca^{2+} channel incorporated into lipid bilayers.** Purified RyR was fused with lipid bilayer and single channel recording and current integral *versus* time were measured as described under "Experimental Procedures." Current integral values are 1138 pA·ms before the addition of peptide 2 (*control*) and 215 pA·ms after addition of peptide 2 (100 nM final concentration). *Horizontal arrows* indicate the closed state of the channel. This experiment is representative of four experiments.

with RyR. The interaction of the cytoplasmic domain of triadin with RyR is inhibited at millimolar [Ca^{2+}]. We show that the cytoplasmic domain of triadin plays a functional role in the regulation of the Ca^{2+} release from SR via the control of the RyR Ca^{2+} channel behavior. Indeed, antibodies directed against the cytoplasmic region of triadin induce both an inhibition of Ca^{2+} release from SR and a decrease of the open probability of RyR Ca^{2+} channel. Moreover, peptide 2 representing the domain of triadin directly involved in the interaction with the RyR can induce *in vitro* the inhibition of the RyR channel activity. These results clearly demonstrate that tria-

din-RyR interaction directly modulates the intrinsic RyR Ca^{2+} channel properties and are in agreement with previous works (32, 33) suggesting that triadin is involved in the control of Ca^{2+} flux from SR.

Recently, Ohkura *et al.* (36) have shown that addition of purified skeletal triadin to the cytoplasmic side of purified RyR incorporated in lipid bilayer induces a complete inhibition of RyR channel activity, whereas addition of triadin to the luminal side does not produce any change in the RyR activity. These authors propose that this regulation of RyR by triadin involves a cytoplasmic domain of triadin. According to our results, the cytoplasmic domain of triadin encompassing residues 18–46 could be a good candidate to mediate this interaction. Therefore we propose that the binding of the A-Nter to its epitope, which is located within the first 17 residues of triadin, stabilizes the interaction of a specific site, located between residues 18 and 46 of triadin, with RyR, leading to the stabilization of the closed state of the RyR Ca^{2+} channel. The involvement of triadin in the control of RyR via the cytoplasmic side also agrees with the results obtained by Liu and Pessah (33) showing the effect of cytoplasmic modulators on both triadin and RyR. However, this interaction of triadin with the RyR has to be strictly regulated. Interestingly, Guo and Campbell (31) have shown that the luminal domain of triadin is able to interact with both RyR and calsequestrin and have proposed that triadin could be the link between these two proteins. Therefore, according to the results presented here, it appears that triadin not only could mediate the control of the RyR Ca^{2+} channel activity by luminal Ca^{2+} but also participate in the regulation of the RyR channel activity by cytoplasmic factors. Moreover, the luminal part of triadin could play a role in the regulation of the inhibitory effect of the cytoplasmic domain, providing a direct link between the luminal Ca^{2+} concentration and the RyR activity. The strict dependence on free cytoplasmic Ca^{2+} concentration of the interaction of the cytoplasmic domain of triadin with RyR, described above, likely explains why Guo and Campbell (31) did not see any interaction of RyR with the fusion protein corresponding to the cytoplasmic domain of triadin, because their experiments were performed in the presence of mM Ca^{2+}. Interestingly the RyR-triadin interaction highlighted in the present work is shown to be inhibited by Ca^{2+} in a range of concentrations in which Ca^{2+} has previously been shown to control Ca^{2+} release from SR as well as ryanodine binding on RyR and therefore the functional state of RyR (7, 35). This effect of Ca^{2+} on the RyR-triadin interaction suggests that the interaction of the cytoplasmic domain of triadin with RyR depends on the RyR conformational state and *vice versa*. Moreover, RyR·triadin complex is stabilized in the presence of Ca^{2+} concentrations corresponding to the cytoplasmic Ca^{2+} level of the resting cell, and strongly disfavored in the presence of Ca^{2+} concentrations corresponding to the $[Ca^{2+}]$ locally reached when RyR Ca^{2+} channels open. Therefore, RyR-triadin interaction, by sensing the $[Ca^{2+}]$ at the RyR channel mouth, could represent *in vivo* an important step of control of the RyR Ca^{2+} channel opening and closing by cytoplasmic Ca^{2+} level. The identification of the region of the ryanodine receptor involved in the interaction with the cytoplasmic domain of triadin will help to understand the process of cross-talk between triadin and RyR. In cardiac SR, the ryanodine receptor has been shown to also interact with an other integral membrane protein called junctin also present in skeletal muscle SR (25, 34). Although junctin and triadin show some similarities (25), the primary sequence of their cytoplasmic domain strongly differs, suggesting that they may have a different function, whereas their luminal domain sharing some association motifs is involved in the interaction with RyR and calsequestrin. Comparison of the effect of cytoplasmic domain of triadin and junctin could help to understand the respective role of these proteins.

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