FKBP12 Modulation of the Binding of the Skeletal Ryanodine Receptor onto the II-III Loop of the Dihydropyridine Receptor

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ABSTRACT In skeletal muscle, excitation-contraction coupling involves a functional interaction between the ryanodine receptor (RyR) and the dihydropyridine receptor (DHPR). The domain corresponding to Thr^{671}-Leu^{690} of the II-III loop of the skeletal DHPR α_1-subunit is able to regulate RyR properties and calcium release from sarcoplasmic reticulum, whereas the domain corresponding to Glu^{724}-Pro^{760} antagonizes this effect. Two peptides, covering these sequences (peptide A_{Sk} and C_{Sk}, respectively) were immobilized on polystyrene beads. We demonstrate that peptide A_{Sk} binds to the skeletal isoform of RyR (RyR1) whereas peptide C_{Sk} does not. Using surface plasmon resonance detection, we show that 1) domain Thr^{671}-Leu^{690} is the only sequence of the II-III loop binding with RyR1 and 2) the interaction of peptide A_{Sk} with RyR1 is not modulated by Ca^{2+} (pCa 9–2) nor by Mg^{2+} (up to 10 mM). In contrast, this interaction is strongly potentiated by the immunophilin FKBP12 (EC_{50} = 10 nM) and inhibited by both rapamycin (IC_{50} = 5 nM) and FK506. Peptide A_{Sk} induces a 300% increase of the opening probability of the RyR1 incorporated in lipid bilayer. Removal of FKBP12 from RyR1 completely abolishes this effect of domain A_{Sk} on RyR1 channel behavior. These results demonstrate a direct interaction of the RyR1 with the discrete domain of skeletal DHPR α_1-subunit corresponding to Thr^{671}-Leu^{690} and show that the association of FKBP12 with RyR1 specifically modulates this interaction.

INTRODUCTION

Excitation-contraction (EC) coupling represents the different steps leading to the contraction of the muscle cell following the depolarization of its plasma membrane by the action potential. Two proteins are responsible for this process, the dihydropyridine receptor (DHPR), located in the plasma membrane, and the ryanodine receptor (RyR), located in the membrane of the sarcoplasmic reticulum (SR). Although a number of similarities can be found between the skeletal RyR type (RyR1) and the cardiac type (RyR2) (Coronado et al., 1994; Otsu et al., 1990) or between the skeletal DHPR and the cardiac type (Catterall, 1991; Campbell and Giles, 1990; Mikami et al., 1989), it is widely accepted that the molecular process of EC coupling strongly differs in cardiac cells from skeletal cells (Rios and Pizarro, 1991; Rios et al., 1991; Schneider and Chandler, 1973). In cardiac muscle cells, the Ca^{2+} influx through the DHPR directly induces the release of Ca^{2+} from the SR via the RyR2 by a calcium-induced calcium release process. In contrast, in skeletal muscle cells, internal charge movement within the DHPR, acting as a voltage sensor, induces a conformational change of RyR1 that leads to its opening and to the release of Ca^{2+} from SR. This difference in EC coupling mechanism between skeletal and cardiac muscle cells has been used by a number of groups to define in more details the molecular determinants of this process. It was first shown by Tanabe and co-authors (1988) and by Knudson and co-authors (1989) that the α_{1S} subunit of the DHPR is at least partially responsible for EC coupling in skeletal muscle. Next, the expression of various chimeric DHPR α_1-subunit in dysgenic myotubes allowed the identification of a 126-amino-acid domain, within the cytoplasmic II-III loop of the α_1-subunit, as being an important determinant for skeletal type EC coupling (Tanabe et al., 1990a,b). Meanwhile, the hypothesis of an interaction between RyR1 and the skeletal DHPR was reinforced by the demonstration of their specific arrangement in the SR and plasma membrane as well as by co-purification and co-immunoprecipitation experiments (Block et al., 1988; Marty et al., 1994). During the last few years, important efforts have been made to determine the domains of both skeletal muscle DHPR and RyR1 involved in this interaction. El-Hayek and collaborators (El-Hayek et al., 1995; El-Hayek and Ikemoto, 1998) identified two discrete domains of the II-III loop, termed domain A (Thr^{671}-Leu^{690}) and domain C (Glu^{724}-Pro^{760}) able to regulate Ca^{2+} release from SR vesicles. Domain A activates SR Ca^{2+} release from and ryanodine binding to SR vesicles whereas domain C antagonizes the effect of domain A. The triggering effect of domain A on Ca release from SR vesicles has since been confirmed by different approaches (Dulhunty et al., 1999; Casarotto et al., 2000; Lamb et al., 2000). Recently, Proenza and collaborators (2000) have shown that expression, in dysgenic mice, of α_1-subunit of skeletal DHPR carrying a scrambled sequence of residues 681–690, part of domain A, led to the recovery of the skeletal type of EC coupling, suggesting that domain A is not directly responsible for EC coupling.

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In parallel, different studies have attempted to identify the domain of RyR1 involved in the interaction with the II-III loop of the DHPR. Molecular interaction studies have shown that RyR1 residues Leu^{922}-Asp^{1112} bind specifically to the DHPR II-III loop (Leong and MacLennan, 1998a,b). Meantime, functional studies of EC coupling in cells expressing RyR chimera, containing different regions of RyR1 and RyR2, lead to the identification of different domains of RyR1, responsible for the interaction with the DHPR, corresponding to the residues 1303–1406 (Yamazawa et al., 1997) and 1635–2636 (Nakai et al., 1998b), respectively.

In parallel, the number of proteins shown to interact with the RyR and to modify its activity has considerably increased (for review see MacKrill, 1999). One of these proteins, the immunophilin FKBP12, is tightly associated with RyR1. FKBP12 is a particularly interesting partner of the RyR for two main reasons. First, its continuous association with highly purified RyR1 and its high affinity for RyR1 (Timerman et al., 1995; Jayaraman et al., 1992) strongly suggest that FKBP12 is constitutively associated with RyR1. Second, different isoforms of FKBP, FKBP12, and FKBP12.6 seem to associate with the skeletal RyR1 and the cardiac RyR2, respectively (Lam et al., 1995; Barg et al., 1997), suggesting that this protein could partially contribute to some of the difference in EC coupling mechanism between these two tissues. Although it has been demonstrated that FKBP12 modifies the biophysical properties of RyR1 channel by stabilizing its full conductance state (Brillantes et al., 1994; Ahern et al., 1997), the exact role of FKBP12 in the control of EC coupling remains to be elucidated.

In the present work, we have used molecular approaches to study the binding of domain A (residue Thr^{671}-Leu^{690}) and domain C (Glu^{724}-Pro^{760}) of the α_{1S}-subunit from the DHPR, with RyR1, the skeletal isoform of RyR. We demonstrate that only the skeletal form of the domain A binds to RyR1 and is able to inhibit the binding of RyR1 with the full-length II-III loop of the skeletal DHPR α_1 subunit. We also demonstrate that the association of FKBP12 with RyR1 is required for the interaction of the domain A with RyR1. In support of this observation, we show that the modulation of RyR1 channel activity by DHPR domain A is completely abolished following FKBP12 depletion from RyR1.

MATERIALS AND METHODS

Isolation of SR vesicles

The skeletal heavy SR vesicles were prepared from rabbit leg and back muscles by differential centrifugation, as previously described (Marty et al., 1994; Anderson et al., 1989).

Purification of RyR

The skeletal SR vesicles were solubilized in the presence of CHAPS, and RyR1 was purified by centrifugation of the solubilized proteins on a sucrose density gradient (Lai et al., 1988). Purified RyR preparations were stored in liquid nitrogen and their concentration determined using the Folin method relative to bovine serum albumin.

Synthesis of peptides

Three peptides named peptide A_{Sk} and peptide A_{Cd}, corresponding to residues Thr^{671}-Lys^{690} and Thr^{795}-Ala^{812} of the II-III loop of the skeletal and cardiac DHPR α_1-subunit, and peptide C_{Sk} corresponding to residues Glu^{724}-Pro^{760} of the skeletal DHPR α_1-subunit were synthesized with an exogenous biotin on the C-terminal amino acid.

The amino acid sequences of these peptides are as follows: peptide A_{Sk}, TSAQKAKAEERKRRKMSRGL; peptide A_{Cd}, TSAQKEEEEEKERKKLARTA; and peptide C_{Sk}, EFESNVNEVKDPYPSADFPGDDEEDEPEIPV.

Synthetic peptides were purified by chromatography, lyophilized, and stored at −20°C. Before use, the peptides were resuspended in sterile water at a concentration of 10 mg/ml. Further dilutions were made in a buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl (buffer T).

Expression and purification of the skeletal α_{1S} II-III loop

cDNA construction

The cDNA coding for the His-II-III_S fusion protein was obtained by subcloning the cDNA sequence of the II-III_S loop into the His-pMR vector. The α_{1S} cDNA (GenBank X05921 rabbit α_{1S}-subunit) was used as a template for II-III_S cDNA amplification, using the following oligonucleotides: forward, 5′-CATGCCATGGCAGTGGACAACCTGGCCGAGGCCGAGAGCCTGACT-3′, and reverse, 5′-GCTCTAGAGTCAGGTGGCGTTGACGATGCGGTGACACAGGACACG-3′.

The PCR product was purified and subcloned into the *Nco*I and *Xba*I sites of the pMR vector. This vector contains a minicistron for high-level expression and a sequence coding for a polyhistidine tail fused to the amino terminus of cloned genes (Arnaud et al., 1997).

Purification

Large BL21 bacteria cultures (400 ml) transformed with the pMR-II-III_S-His plasmid were grown at 37°C under agitation until an OD_{600} of 0.6 was reached. Protein synthesis was induced by adding 1 mM isopropyl-1-1-thio-β-D-galactopyranoside to the culture media for 4 h. The bacteria were then pelleted (5000 rpm for 10 min on a Centrikon A 6.14 rotor, Kontron Instruments, Paris, France) and resuspended in 10 ml of Tris-buffered saline (TBS; 150 mM NaCl, 25 mM Tris, pH 7.4 with HCl) supplemented with 1 mM benzaminide, 0.23 mM phenylmethylsulfonyl fluoride. The cells were sonicated four times on ice for 30 s and the sample clarified by centrifugation at 14,000 × *g* (AM3815 rotor, Jouan MR22i) for 15 min. The supernatant was kept on ice. Five milliliters of iminodiacetic acid coupled onto Sepharose beads (Sigma Chemical Co., St. Louis, MO) were added to a disposable polypropylene column leading to a final bed volume of 2.5 ml. The column was washed with 3 vol of H_2O, charged with 5 vol of $NiSO_4$ (50 mM), and equilibrated with 3 vol of loading buffer containing (in mM): 40 imidazole, 500 NaCl, 50 Tris-HCl, pH 7.9. The sample was then charged onto the column and washed with 30 ml of the same buffer. The elution was performed with 15 ml of elution buffer (in mM: 200 imidazole, 500 NaCl, 50 Tris-HCl, pH 7.9). One-milliliter fractions were collected during elution. Fractions were analyzed by electrophoresis on a 3–12% SDS-polyacrylamide gel, and protein bands were visualized by staining with Coomassie Blue.

Immobilization on streptavidin biomagnetic beads

One milligram of streptavidin Dynabeads (Dynal, Compiègne, France), washed three times with buffer T, were incubated for 30 min at room

temperature in the presence of biotinyled peptide A (50 μg/ml). The beads were then washed three times with buffer T and incubated with purified RyR (100 nM final concentration) for 1 h at room temperature. Purified RyR that did not interact with the immobilized peptide was removed by washing the beads twice with buffer T. The proteins bound to the immobilized peptide were then eluted by boiling the beads for 5 min in 2.5% SDS, separated on polyacrylamide gel, and analyzed by Western blot using antibodies directed against the RyR.

Western blot analysis

Proteins were separated by electrophoresis on a 5–20% polyacrylamide gel and transferred on Immobilon P sheet (4 h at 1 A). After saturation with 0.1% PBS-Tween and 4% milk (Biorad, Richmond, CA), the membrane was incubated overnight at 4°C with anti-RyR antibodies (1/10,000 in PBS-Tween). After several washes, the membrane was incubated for 3 h at room temperature with goat anti-rabbit horseradish-peroxidase-conjugated antibodies (1/40,000 in PBS-Tween, Jackson ABQ). The immunolabeling was measured by chemiluminescence technique (Renaissance, NEN) using x-ray films (hyperfilm βmax, Amersham, Pharmacia Biotech, Les Vlis, France).

Real-time surface plasmon resonance measurements

Real time surface plasmon resonance (SPR) experiments were performed on a BIAcore biosensor system 1000 (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were carried out at 25°C.

Peptides were immobilized on the sensor chip surface via an electrostatic bond ($K_d = 10^{-15}$ M) between their biotin and the surface-attached streptavidin. Successful immobilization was evidenced by an increase in resonance units of the order of 800 RU.

Recombinant II-III loop from the skeletal DHPR α_{1S}-subunit was immobilized on anti-His antibodies (Qiagen, Chatsworth, CA) covalently immobilized on the sensor chip. Anti-His antibodies were immobilized on the sensor chip via amino groups according to the manufacturer's procedure.

Purified RyR, previously dialyzed overnight at 4°C against a buffer containing (in mM) 10 HEPES, 150 NaCl, pH 7.4, was injected at various concentrations in similar buffer containing 0.005% polysorbate (running buffer). Nonspecific interaction was measured by passing RyR over a control surface saturated with biotin.

To measure the apparent affinity of the peptide A_{Sk}-RyR1 interaction, RyR1 was incubated for 30 min at room temperature in the presence of various concentrations of peptide A_{Sk} before injection on a sensor chip coated with immobilized peptide A_{Sk}.

Experiments with FKBP12 or rapamycin

Purified RyR (5 nM) was incubated at room temperature for 45 min in the presence of FKBP12 (human recombinant FKBP12, Sigma) or rapamycin (Sigma).

Rapamycin (1 mM) was stored at −20°C in ethanol:dimethylformamide (8:2) and diluted before use in 10 mM HEPES, pH 7.4, 150 mM NaCl. FKBP12 was diluted in distilled water and stored at 4°C.

Preparation of RyR1 depleted of FKBP12

To dissociate FKBP12 from the RyR, heavy sarcoplasmic reticulum vesicles were incubated in the presence of 5 fold molar excess of FK506, at 37°C for 30 min. FKBP12 depleted HSR vesicles were then collected by centrifugation and used for the preparation of FKBP12 depleted RyR as described by Barg et al. (1997). FK-506 was a generous gift from Fujisawa Co. (Munich, Germany).

RyR Ca^{2+} channel reconstitution and single-channel recording analysis

Measurements of channel activity were carried out using solubilized RyR1 incorporated into planar lipid bilayer. The bilayers were formed using phosphatidylethanolamine, phosphatidylserine, and L-phosphatidylcholine in a ratio of 5:4:1 dissolved in *n*-decane up to the final lipid concentration of 20 mg/ml (Herrmann-Frank et al., 1996). Bilayers were formed across a 120- or 250-μm aperture of a nolrene cap using a symmetrical buffer solution (250 mM KCl, 100 μM EGTA, 150 μM $CaCl_2$, 20 mM PIPES, pH 7.2). The chamber into which the small aliquot of solubilized RyR1 was added is defined as the *cis* (cytoplasmic) side, whereas the other chamber, labeled as the *trans* (luminal) side, was kept on ground potential. After successful incorporation of RyR1 channel, free calcium concentration in the *cis* chamber was adjusted to 10 μM by addition of EGTA. At the end of the measurement, ryanodine or ATP was applied to the *cis* chamber to verify the orientation of the incorporated RyR1 being tested. Current signals were filtered at 1 kHz using an 8-pole low-pass Bessel filter and digitized at 3 kHz using an Axopatch 200 amplifier and pClamp 6.03 software (Axon Instruments, Foster City, CA). Total recording time in each experiment was 5–10 min for any experimental condition tested. After changing conditions, at least 5 min were allowed for equilibration, which appeared to be enough to reach the new equilibrium of the parameters. Single-channel measurements were carried out at 20–22°C; the free Ca^{2+} concentration was calculated using the computer program and affinity constants published by Fabiato (1988). Open probabilities were calculated using the common 50% criteria with a medial dead zone of 5%. Current amplitude distribution was analyzed using Origin (Microcal Software, Northampton, MA). Values of the open probability and of the mean open time are expressed as means ± SE.

RESULTS

Binding of RyR1 on peptide A_{Sk}

Peptides corresponding to residues Thr^{671}-Leu^{690} (peptide A_{Sk}) and Glu^{724}-Pro^{760} (peptide C_{Sk}) of the α_1-subunit of the skeletal DHPR, and to the residues Thr^{795}-Ala^{812} (peptide A_{Cd}) of the cardiac DHPR were synthesized with a biotin attached to the C-terminal end as described in Materials and Methods. To study their interaction with purified RyR1, these peptides were immobilized on polystyrene beads. Back titration of the unreacted peptide was performed by ELISA technique, using antibodies directed against biotin, and shows that, under the conditions used (beads/peptide = 50 w/w), all the biotinylated peptide was immobilized on the beads (data not shown). The beads coated with peptide $A_{Sk,}$ peptide C_{Sk}, or peptide A_{Cd} were then incubated with purified RyR1, washed with buffer, and treated with SDS to elute proteins that reacted with the peptide. These proteins were then analyzed by SDS-PAGE or Western blot using antibodies directed against RyR1. Fig. 1 *A* shows the SDS-PAGE analysis of purified RyR1 preparation and of the proteins present in the liquid phase at the different steps of an experiment of interaction between RyR1 and peptide A_{Sk}. Lane 2 shows the disappearance of RyR1 in the medium after incubation with the beads, indi-

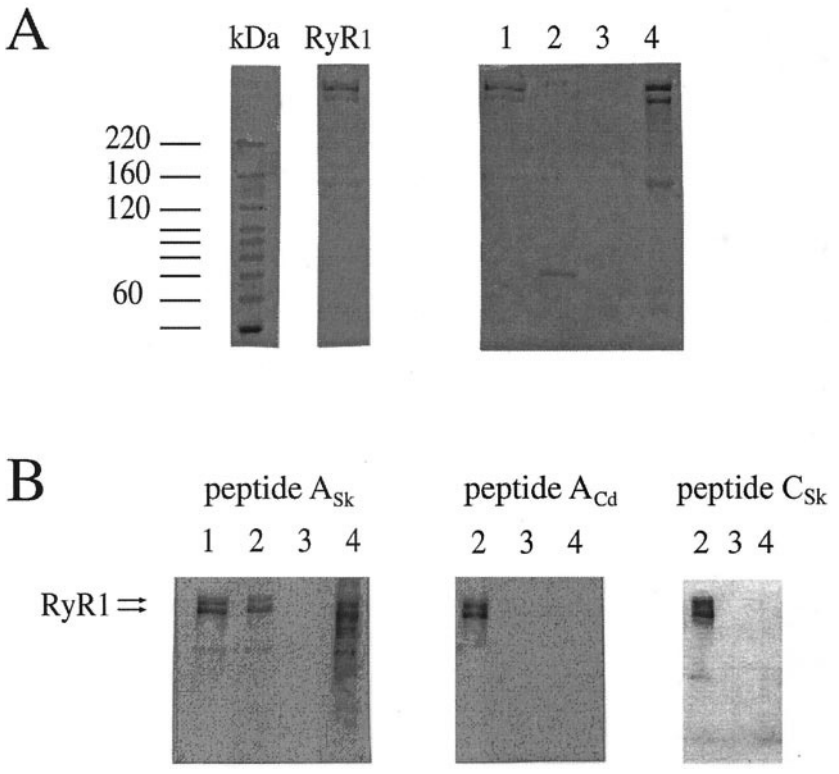


FIGURE 1 Interaction of RyR1 with the different domains of α_{1S} II-III loop. Peptide A_{Sk} and peptide A_{Cd} and peptide C_{Sk} were immobilized on magnetic streptavidin beads as described in Materials and Methods. Purified RyR1 (100 nM in 10 mM HEPES, pH 7.4, 150 mM NaCl) was added to an equal amount of peptide-coated beads. After incubation for 1 h at room temperature, the beads were washed three times with the same buffer and the bound proteins eluted by boiling in the presence of SDS. (*A*) Coomassie blue-stained polyacrylamide gel (5–15%) of purified RyR1 preparations (1 μg) and of the supernatant at different steps of the experiment: lane 1, RyR1 preparation before incubation with peptide A_{Sk}-coated beads; lane 2, supernatant after incubation; lane 3, supernatant after the third wash; lane 4, supernatant after treatment of the beads with SDS. (*B*) Immunoblot analysis, with antibodies directed against RyR1, of the supernatant at different steps of the interaction experiments: left part, RyR1 with peptide A_{Sk}; middle part, RyR1 with peptide A_{Cd}; right part, RyR1 with peptide C_{Sk}. Lane 1, RyR1 preparation before incubation with peptide A coated beads; lane 2, supernatant after incubation; lane 3, supernatant after the third wash; lane 4, supernatant after treatment of the beads with SDS.

cating the interaction of RyR1 with peptide A_{Sk}. Indeed, RyR1 bound to the peptide is recovered after treatment of the beads with SDS, as shown on lane 4. The different bands appearing on lane 4, and in a smaller amount in lane 1, are due to a proteolytic degradation of RyR1 caused both by the incubation at room temperature and by the 5-min boiling step necessary for the elution from the beads. Lane 3 shows the absence of RyR1 in the washing solution, indicating that the peptide A_{Sk}-RyR1 interaction is preserved during this step. Absolutely no interaction of RyR1 with the naked beads was observed (not shown). Fig. 1 *B* represents the Western blot analysis of similar experiments performed with either peptide A_{Sk} (left part), peptide A_{Cd} (middle part), or peptide C_{Sk} (right part), immobilized on the beads and incubated with RyR1. On this figure, the presence of RyR1 was revealed by immunolabeling with specific antibodies. These results clearly show that peptide A_{Sk} binds to RyR1 (lane 4, left part) whereas, in the same conditions, no RyR1 binding can be detected on peptide A_{Cd} or peptide C_{Sk} (lane 4, middle and right part).

The interaction of peptide A with purified RyR1 was then analyzed in greater detail using surface plasmon resonance (SPR) detection. This technique measures the real-time association and dissociation of molecules on a sensor surface. For this purpose, equivalent amounts of peptide A_{Sk}, peptide C_{Sk}, or peptide A_{Cd} were immobilized on a streptavidin-coated sensor chip, as described in Materials and Methods. Fig. 2 *A* (*upper panel*) represents the sensorgrams of the interaction of RyR1 with peptide A_{Sk} (*trace d*), peptide C_{Sk} (*trace b*), and peptide A_{Cd} (*trace c*). Histograms representing the average amplitude of these interactions are presented on Fig. 2 *B*. These results show that only peptide A_{Sk} interacts with RyR1. This interaction was inhibited when the purified RyR1 was incubated in presence of peptide A_{Sk} (from 15 nM to 3 μM) (Fig. 2 *A*, *lower panel*) before the injection on immobilized peptide A_{Sk}. Half-inhibition (IC_{50}), reflecting the apparent affinity of peptide A_{Sk} for RyR1 in solution, is observed at 130 nM peptide A_{Sk}.

In another set of experiments, the entire II-III loop of the skeletal DHPR α_1-subunit was immobilized on a sensor

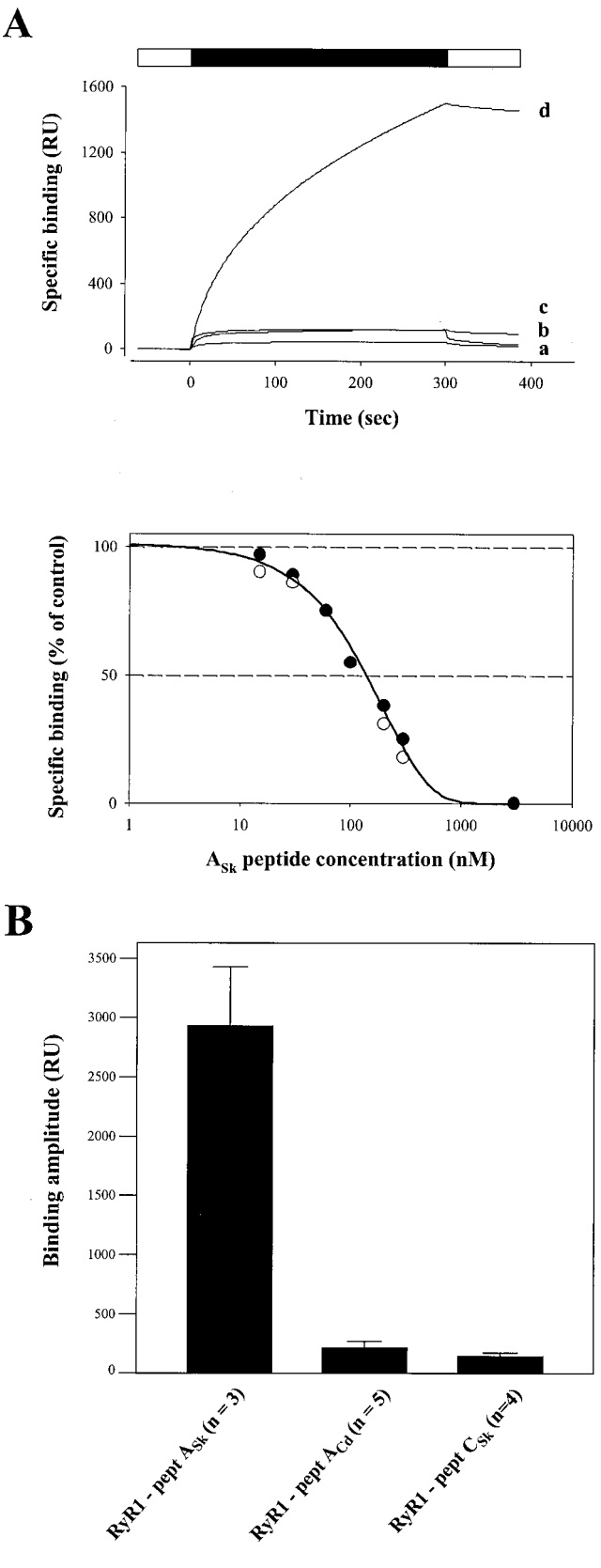


FIGURE 2 Real-time SPR detection of RyR1 binding on immobilized domains of the II-III loop. (*A*, *upper panel*) SPR sensorgrams of the interaction of purified RyR1 (6.5 nM in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% polysorbate 20, running buffer) with biotin (*trace a*), peptide C_{Sk} (*trace b*), peptide A_{Cd} (*trace c*), or peptide A_{Sk} (*trace d*). An equivalent amount of each peptide was immobilized on different flow cell as described in Materials and Methods. The shaded bar above the plot indicates the injection of RyR1, and the empty bars denote running buffer alone. (*Lower panel*) Dose-response relationship for inhibition of the binding of RyR1 on immobilized peptide A_{Sk} by free peptide A_{Sk}. Purified RyR1 was incubated in presence of peptide A_{Sk} (various concentrations in running buffer) for 30 min at room temperature before injection in a flow cell containing immobilized peptide A_{Sk}. Residual binding amplitude is expressed in percent of the binding amplitude measured when RyR1 was incubated in the absence of peptide A_{Sk}. The continuous curve was obtained by fitting the data according to the equation: residual binding = $1/(1 + [A_{Sk}]/EC_{50})$. Opened and closed symbols represent experiments done with two different RyR1 preparations. (*B*) Amplitude histogram of the specific interaction signal of RyR1 (15 nM in running buffer) with peptide A_{Sk}, peptide A_{Cd}, and peptide C_{Sk}. The amplitude of the binding signal was measured after 5 min of interaction. Nonspecific binding was measured by injection of RyR1 on flow cells containing immobilized biotin.

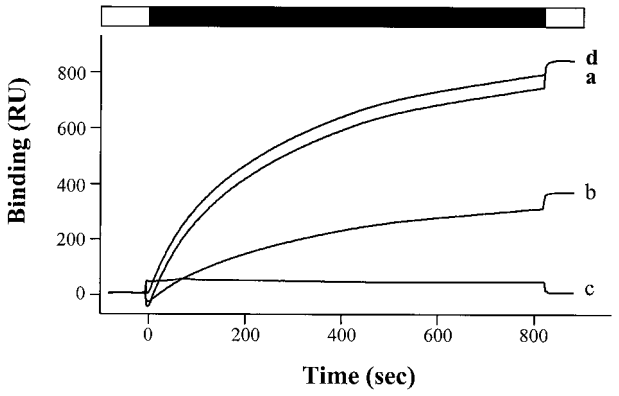


FIGURE 3 Peptide A_{Sk}, but not peptide C_{Sk}, inhibits the binding of RyR1 on the full-length II-III loop of skeletal DHPR α_{1S}-subunit. Purified RyR1 (200 nM in running buffer) was injected on the recombinant II-III loop of the DHPR α_{1S}-subunit immobilized on sensor chip (see Materials and Methods). Before injection, purified RyR1 was incubated for 1 h in the absence of (*trace a*) or presence of 380 nM peptide A_{Sk} (*trace b*), 38 μM peptide A_{Sk} (*trace c*), or 38 μM peptide C_{Sk} (*trace d*). These curves are representative of three different experiments. The shaded bar above the plot indicates the injection of RyR1; empty bars denote running buffer alone.

chip and tested for the interaction with RyR1. The II-III loop was expressed as a His-tag fusion protein and immobilized on the sensor chip via anti His-tag antibodies covalently immobilized on the sensor chip. Fig. 3 shows the sensorgram of the interaction of purified RyR1 with immobilized II-III loop (*trace a*). This interaction was inhibited when RyR1 was incubated in the presence of peptide A_{Sk} (*traces b* and *c*) before the injection on the immobilized II-III loop. Incubation of RyR1 in presence of an excess of peptide A_{Sk} (38 μM, *trace c*) completely prevents its binding on the immobilized II-III. In contrast, incubation of RyR1 in the presence of an excess of peptide C_{Sk} (38 μM, *trace d*) did not alter the interaction of RyR1 with the immobilized II-III loop. The slight shift between traces a

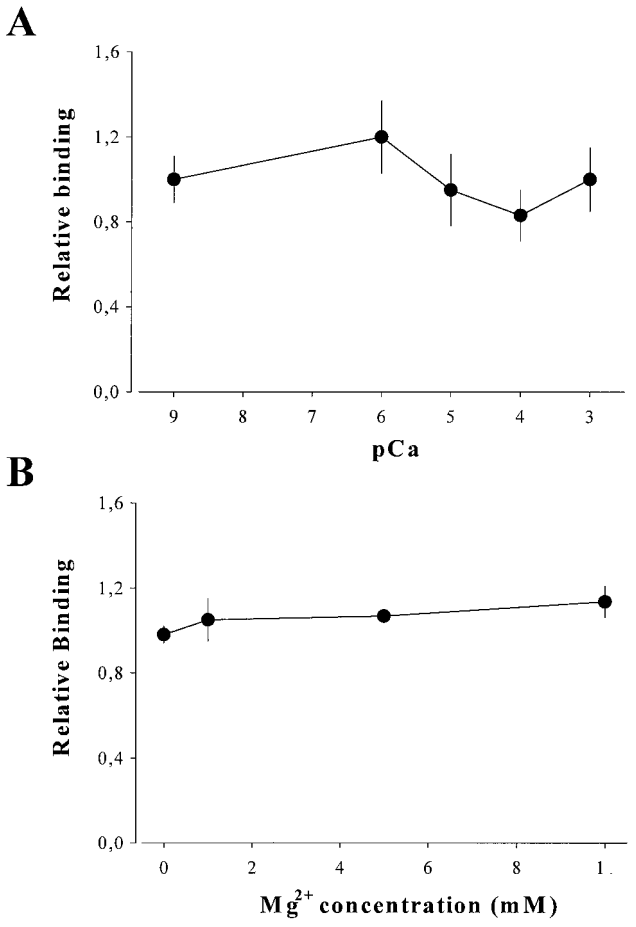


FIGURE 4 RyR1-peptide A_{Sk} interaction is independent of the Ca^{2+} and Mg^{2+} concentrations. Purified RyR1, 5 nM in 10 mM HEPES, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.005% polysorbate 20, and various $CaCl_2$ concentrations (*A*) or in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% polysorbate 20, and various $MgCl_2$ concentrations (*B*) was injected into a flow cell containing immobilized peptide A_{Sk}. The amplitude of the specific binding signal was measured after 5 min. Each point represents the average ± SD of at least four experiments.

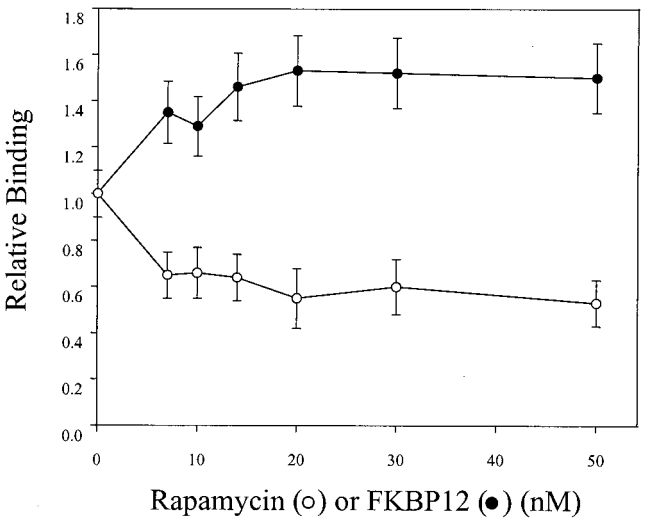


FIGURE 5 Effect of FKBP12 and rapamycin on the binding of RyR1 on peptide A_{Sk}. RyR1 (5 nM in running buffer) was incubated for 45 minutes at room temperature in the presence of various concentrations of rapamycin (○) or human recombinant FKBP12 (●) and then injected into a flow cell containing immobilized peptide A_{Sk}. The amplitude of the specific binding signal was measured after 5 min. Each point represents the average value ± SD of at least seven experiments.

and d is due to the presence in the running buffer of the peptide C_{Sk} but does not represent a significant change in the binding signal amplitude.

Regulation of the binding of RyR1 on peptide A_{Sk}

We therefore examined the possible regulation of RyR1-peptide A_{Sk} interaction by different effectors of the EC coupling such as Ca^{2+} and Mg^{2+}. Fig. 4 *A* represents the amplitude of RyR1 binding on peptide A_{Sk} in presence of increasing concentrations of free Ca^{2+} and shows that the interaction is not modified by changes in Ca^{2+} concentration. No significant change of the association or dissociation rates was observed in the range of free Ca^{2+} concentrations studied. Similarly, RyR1 binding on peptide A_{Sk} was not modified by increasing Mg^{2+} concentration from 0 to 10 mM (Fig. 4 *B*).

In a second set of experiments, we investigated the role of the FKBP12 in the regulation of RyR1-peptide A_{Sk} interaction. Two drugs, rapamycin and FK506, are known to bind to FKPB12 and induce its dissociation from RyR1. Using the SPR approach, we measured RyR1-peptide A_{Sk} interaction after incubation of RyR1 with rapamycin or with FKBP12. The results presented on Fig. 5 show that preincubation of RyR1 with rapamycin (open circles) induces an inhibition of its binding on peptide A_{Sk} (IC_{50} in the low nanomolar range). Similar results were obtained using FK506 instead of rapamycin (not shown). Full binding inhibition was not reached, presumably because these drugs are unable to remove all the RyR1-associated FKBP12. Conversely, preincubation of RyR1 with increasing concentrations of recombinant FKBP12 (Fig. 5, closed circles) potentiates its binding on peptide A_{Sk} (EC_{50} = 10 nM). This effect of FKBP12 was completely abolished when FKBP12 was incubated with rapamycin before the incubation with RyR1 (data not shown). These results clearly demonstrate the involvement of FKBP12 in the formation of the interaction RyR1-peptide A_{Sk}. The effect of FKBP12 addition to the purified RyR1 suggests that the purified RyR1 preparation may contain RyR1 molecules associated with 0, 1, 2, 3, or 4 FKBP12, in agreement with the results obtained by different groups (Jayaraman et al., 1992; Timerman et al., 1995). These results suggest that FKBP12 may serve as a link between domain A of the DHPR α_1-subunit and RyR1. To test this hypothesis, we investigated the binding of

FKBP12 on peptide A_{Sk}. Recombinant FKBP12 (up to 1 μM) was injected on a sensor chip coated with peptide A_{Sk}. Because of the low molecular weight of FKBP12, which could result in a small binding signal, anti-FKBP12 antibodies were injected just after recombinant FKBP12. No binding signal was observed after injection either of FKBP12 or of the antibodies (not shown). The effect of FKBP12 is therefore not mediated through a direct interaction between FKBP12 and domain A of the DHPR.

Effect of peptide A_{Sk} on RyR1 Ca^{2+} channel behavior

To investigate the functional aspect of the interaction RyR1-peptide A_{Sk}, we studied the effect of peptide A_{Sk} on the behavior of purified RyR1 reconstituted in artificial lipid bilayer as described in Materials and Methods. Fig. 6 *A* represents the single-channel recording of purified RyR1 incorporated in lipid bilayer. Sixty to eighty percent of the reconstituted channels were ryanodine sensitive and presented only two peaks on the current histogram. This last criterion is a widely accepted sign that the RyR1 channel is in its native state associated to all four FKBP12 molecules. The addition of peptide A_{Sk} to the *cis* chamber induces an increase of the open probability of RyR from 0.143 $\pm$ 0.052 (SE, $n = 8$) to 0.482 $\pm$ 0.131 (SE, $n = 5$) in the presence of 26 μM peptide A_{Sk}, whereas no significant change of the mean open time was observed. This activation by peptide A_{Sk} was independent of the holding potential and was observed at positive or negative holding potential equally. In a second set of experiments (Fig. 6 *B*), we measured the influence of FKBP12 removal on the effect of peptide A_{Sk} on RyR1 behavior. For this purpose, RyR1 was purified from SR vesicles depleted of FKBP12 by treatment with FK506 as described in Materials and Methods. The efficiency of the depletion is objectified on the histograms presented on Fig. 5 *B* by the fact that depleted RyR1 Ca^{2+} channel opens predominantly to subconductance levels, in contrast to what is observed for the nondepleted RyR1 (Fig. 6 *A*). This characteristic of FKBP12-depleted RyR1 was previously described by several groups (Brillantes et al., 1994; Ahern et al., 1997) and currently represents the most established electrophysiological effect of FKBP12 depletion. The presence of five peaks on the current histogram suggests that FKBP12 depletion is complete on the recorded channel. Full depletion was, however, not reached on each channel, and those recordings that did not possess the five characteristic conductances were discarded. Fig. 6 *B* shows that, in these conditions, the addition of peptide A_{Sk} (up to 26 μM) has no effect on the channel behavior of FKBP12-stripped RyR1, with Po values being 0.312 $\pm$ 0.178 (SE, $n = 5$) and 0.327 $\pm$ 0.158 (SE, $n = 6$) in the control and in the presence of 26 μM peptide A_{Sk}, respectively. No change in the pattern of subconductance levels of stripped RyR1 was observed in the absence or the presence of peptide A_{Sk} (Fig. 6 *B*). To check that normal RyR as well as FKBP12-depleted RyR is still responsive to regular effectors, ATP was added after the addition of the peptide A_{Sk}. Traces c and f and corresponding histograms show a clear activation of the normal RyR and the FKBP12 depleted RyR by addition of ATP. Similar effects of Ca^{2+} and ryanodine were also observed on normal and FKBP12-depleted RYR1 (not shown). To test the reversibility of the effect of FK506 on the interaction of RyR1 and peptide A_{Sk}, FKBP12-depleted RyR1 was incubated in presence of an excess of recombinant FKBP12 before injection of the peptide A_{Sk}. After incubation with FKBP12, RyR1 channel behavior is again characterized by predominant single opening state (not shown). Addition of peptide A_{Sk} on the reconstituted FKBP12-RyR1 complex induces an increase of the open probability of RyR1 from 0.178 ($n = 3$) to 0.496 ($n = 3$) in the presence of 26 μM peptide A_{Sk}, clearly demonstrating the reversibility of the FKBP12 depletion effect on RyR1-peptide A_{Sk} interaction.

We also tested the effect of peptide A_{Cd} on RyR1-FKBP12 activity. Under conditions similar to those described for Fig. 6 *A*, addition of peptide A_{Cd} (up to 26 μM) to the *cis* chamber did not induce any modification of the RyR1 behavior: Po values were 0.223 $\pm$ 0.103 (SE, $n = 6$) for the control conditions and 0.229 $\pm$0.124 (SE, $n = 4$) in the presence of 26 μM peptide A_{Cd} (data not shown).

DISCUSSION

Several studies have previously shown that the cytoplasmic loop linking the repeat II and III of the DHPR α_1-subunit plays a critical role in the control of the EC coupling process (Tanabe et al., 1990b). This region of the DHPR therefore represents a good candidate for interactions with the RyR1. Two domains of this loop, domain A and domain C, respectively, have been postulated to be involved in the control of EC coupling. However, the roles of each of these domains as well as the way they interact with RyR1 are still debated. Domain A activates Ca^{2+} release from SR vesicles and increases RyR1 channel open probability whereas domain C itself does not affect Ca^{2+} release but is able to antagonize the effect of domain A. On the other hand, electrophysiological studies of the EC coupling have shown that domain C is essential in determining the type of EC coupling whereas domain A does not seem to be directly involved in this process.

In this work, we studied the direct interaction of each of these domains with RyR1 and illustrate several new points. First, the domain of the skeletal DHPR α_{1S}-subunit encompassing residues Thr^{671}-Leu^{690} (domain A_{Sk}) binds to RyR1. Under the same conditions, the corresponding domain of the cardiac DHPR α_1-subunit (domain A_{Cd}, residues Thr^{795}-Ala^{812}) does not show any interaction with RyR1. Second, the domain of the skeletal DHPR α_{1S}-subunit encompassing residues Glu^{724}-Pro^{760} (domain C_{Sk})

does not bind to RyR1. Third, domain A_{Sk} but not domain C_{Sk} completely inhibits the interaction of RyR1 with the full-length II-III loop of the skeletal DHPR α_{1S}-subunit. Fourth, the binding of domain A_{Sk} to RyR1 is strongly dependent on the interaction of RyR1 with the immunophilin FKBP12. The association RyR1 domain A_{Sk} is selectively antagonized by the immunosuppressive drugs rapamycin and FK506 and potentiated by addition of recombinant FKBP12. We also show that Ca^{2+} or Mg^{2+} does not regulate the association between domain A_{Sk} and RyR1. Finally, the interaction of domain A_{Sk} with RyR1 induces a modification of the RyR1 Ca^{2+} channel behavior characterized by an increase of the open probability, an effect that is absent when FKBP12 is dissociated from the RyR1.

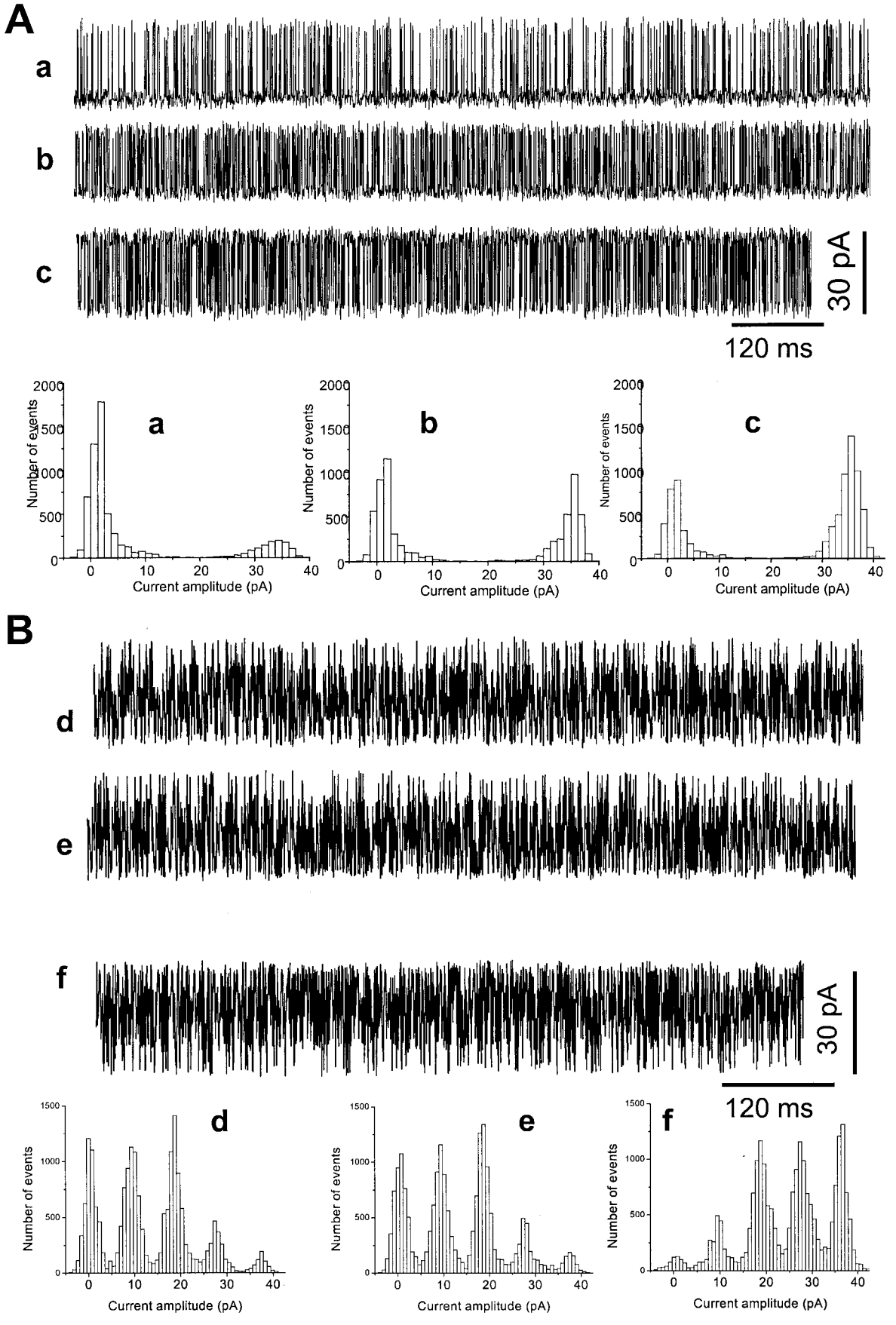
From these results we can conclude that domain A represents the major binding site, if not the only one, of the II-III loop of the α_{1S}-subunit of the DHPR to RyR1. This interaction is strictly dependent of the DHPR isoform because no interaction of the corresponding domain of the cardiac DHPR with RyR1 was observed. The fact that Ca^{2+} or Mg^{2+} does not modulate this interaction suggests that these proteins could be constitutively linked under physiological conditions.

FKBP12, a member of the immunophilins family, has been shown to be tightly associated with RyR1 (K_d in the range of 6 nM), with a stoichiometry of four FKBP12 per tetramer of RyR1 (Timerman et al., 1993. 1995). The immunosuppressive drugs rapamycin and FK506 induce a dissociation of FKBP from RyR. Several groups have shown that FKBP12 modifies the calcium channel gating behavior of RyR1, suggesting that FKBP12 plays a role in the control of the conformational state of the ryanodine receptor (Brillantes et al., 1994; Barg et al., 1997; Ahern et al., 1997). More recently Dulhunty and collaborators (1999) have shown that the ability of peptide A_{Sk} to activate RyR1 is reduced when SR membrane vesicles are treated with rapamycin. We show here that nanomolar concentrations of FKBP12 strongly potentiate the association of skeletal DHPR domain A with RyR1, whereas rapamycin or FK506 inhibits this interaction. Because no direct binding of the FKBP12 on domain A_{Sk} was observed, the inhibition of the RyR1-domain A interaction by rapamycin is likely to be due to allosteric changes of RyR1 initiated by FKBP12 removal. This suggests that FKBP12 stabilizes RyR1 in a conformational state that allows its interaction with the domain A. Using purified RyR1 incorporated in artificial bilayer, we show that the complex RyR1-domain A_{Sk} is characterized by a higher opening probability of the RyR1 Ca^{2+} channel. In complete coherence with what was observed for the interaction of RyR1-domain A, the effect of domain A_{Sk} on the open probability of RyR1 was lost when the FKBP12 is dissociated from the RyR1.

These results clearly demonstrate the important role of FKBP12 in the formation of the protein complex that involves RyR1 and the skeletal DHPR, which is a prerequisite for the establishment of EC coupling in skeletal muscle cells. Interestingly, Lamb and Stephenson (1996) have shown that, in rat skinned skeletal myofibrils, FK506 and rapamycin led to a progressive loss in the depolarization-induced Ca^{2+} release from SR although they had no effect on Ca^{2+} release induced by caffeine. However, skeletal muscle cells of mice lacking FKBP12 do not show significant histological or electron-microscopy-detectable abnormalities (Shou et al., 1998). Although these results question the exact role of FKBP12 in the skeletal muscle cell, they might suggest that FKBP12 as well as the interaction of domain A with RyR1 are involved in a fine control of the EC coupling process during the long-term evolution of the skeletal muscle cells.

Domain C has been shown to antagonize the effect of domain A on depolarization-induced Ca release from SR vesicle or in skinned fiber but is not able to modify, by itself, the Ca release induced by caffeine (Lamb et al., 2000; Saiki et al., 1999; El-Hayek et al., 1995). Electrophysiological studies have shown that domain C is important for the control of EC coupling (Nakai et al., 1998a). Our results, showing that this domain does not interact directly with RyR1, prompt us to propose that the domain C regulates RyR1 by interacting with another protein of the complex or with another domain of the DHPR. This hypothesis could explain the apparent discrepancies in the identification of the domain of RyR1 involved in the interaction with the II-III loop and those involved in the regulation of EC coupling. Indeed, molecular interactions studies have shown that residues 922-1112 of RyR1 bind to the entire II-III loop of the α_{1S} DHPR (Leong and MacLennan, 1998), whereas electrophysiological studies have identified the residues

FIGURE 6 Effect of peptide A_{Sk} on the RyR channel activity and effect of the depletion of FKBP12 from the channel complex. Control and FKBP12-depleted solubilized RyR1 Ca^{2+} channel complex was incorporated into a lipid bilayer and channel current measured under voltage clamp conditions, as described in Materials and Methods. Holding potential was +60 mV: *cis* side [Ca^{2+}] was 10 μM, and charge carrier was K^+ (250 mM *cis* side). Channel openings are represented by upward deflections. Current traces were analyzed for Po, mean open time and amplitude distribution. (*A*) Representative current trace were recorded in the absence of peptide A_{Sk} (*a*, serves as a control) and in the presence of 26 μM peptide A_{Sk} (*b*) or 26 μM of peptide A_{Sk} and 2 mM ATP (*c*). Closed states of the channel are marked on the left margin and calibration current bar exhibited on the bottom of the panel. Current-amplitude distributions obtained under similar conditions are shown in the lower part of the upper panel. (*B*) Representative current traces recorded in the absence of peptide A_{Sk} (*d*, serves as control) and in the presence of 26 μM peptide A_{Sk} (*e*) or 26 μM peptide A_{Sk} and 2 mM ATP (*f*) using FKBP12-depleted RyR1 incorporated into the lipid bilayer. Current-amplitude distributions obtained under identical conditions are shown in the lower part of this panel.


A
a
b
c
30 pA
120 ms
Number of events
Current amplitude (pA)
a
b
c
B
d
e
f
30 pA
120 ms
d
e
f

1303–1406 (Yamazawa et al., 1997) or 1635–2636 (Nakai et al., 1998b) as being involved in the control of EC coupling. Therefore domain A_{Sk}-RyR1 interaction could involve the residues 922-1112 of RyR1 whereas domain C could interact with another domain of the DHPR, or another protein, itself interacting with the residues 1303–1406 or 1635–2636 of RyR1. Other domains of the α_{1S} DHPR subunit such as the C-terminal domain (Slavik et al., 1997) and the III-IV cytoplasmic loop (Leong and MacLennan, 1998b), have been shown to regulate or to interact directly with RyR1 and could therefore represent a potential interacting site with domain C. Recently, Avila and collaborators (2001) have shown that a single mutation (I4898T) in a putative intra-luminal region of RyR1, different from the RyR1 region previously shown to be important for EC coupling, completely abolishes the EC coupling in skeletal muscle cell. These results strongly suggest that several domains of the RyR1, as well as of the DHPR, could control the fine-tuning of the EC process in skeletal muscle.

By allowing the study of the interaction step per se between different domains of the DHPR and RyR, the different approaches developed in the present work should be very useful in determining the specific role of these different domains in the formation and the regulation of the RyR-DHPR complex.

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