Subconductance States in Single-Channel Activity of Skeletal Muscle Ryanodine Receptors After Removal of FKBP12

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ABSTRACT FKBP12 was removed from ryanodine receptors (RyRs) by incubation of rabbit skeletal muscle terminal cisternae membranes with rapamycin. The extent of FKBP12 removal was estimated by immunostaining Western blots of terminal cisternae proteins. Single FKBP12-depleted RyR channels, incorporated into planar lipid bilayers, were modulated by Ca^{2+}, ATP, ryanodine, and ruthenium red in the *cis* chamber and opened frequently to the normal maximum conductance of ~230 pS and to substate levels of ~0.25, ~0.5, and ~0.75 of the maximum conductance. Substate activity was rarely seen in native RyRs. Ryanodine did not alter the number of conductance levels in FKBP12-depleted channels but, at a membrane potential of +40 mV, reduced both the maximum and the substate conductances by ~50%. FKBP12-stripped channels were activated by a 10-fold-lower [Ca^{2+}] and inhibited by a 10-fold-higher [Ca^{2+}], than RyRs from control-incubated and native terminal cisternae vesicles. The open probability (P_o) of these FKBP12-deficient channels was greater than that of control channels at 0.1 μM and 1 mM *cis* Ca^{2+} but no different at 10 μM *cis* Ca^{2+}, where channels showed maximal Ca^{2+} activation. The ~0.25 substate was less sensitive than the maximum conductance to inhibition by Ca^{2+} and was the dominant level in channels inhibited by 1 mM *cis* Ca^{2+}. The results show that FKBP12 coordinates the gating of channel activity in control and ryanodine-modified RyRs.

INTRODUCTION

The ryanodine receptor (RyR), a high-conductance Ca^{2+} channel located in the terminal cisternae (TC) of skeletal muscle sarcoplasmic reticulum (SR), projects into the junctional gap between the transverse tubules and the SR (Block et al., 1988; Lai et al., 1988; Fleischer and Inui, 1989). Depolarization of the transverse tubules activates the dihydropyridine receptor (DHPR), initiating a signal that is transmitted to the RyR, causing it to open and to release Ca^{2+} into the myoplasm, thereby triggering contraction. The signal transmission from the DHPR to the RyR, which is not fully understood, is known as excitation–contraction (E-C) coupling (Rios et al., 1992; Dulhunty, 1992; Melzer et al., 1995). The RyR is closely associated with a number of proteins in the TC, including triadin (Caswell et al., 1991; Guo and Campbell, 1995), calsequestrin (Collins et al., 1990; Ikemoto et al., 1989), calmodulin (Menegazzi et al., 1994; Tripathy et al., 1995) and FKBP12 (Jayaraman et al., 1992; Timerman et al., 1993), many of which have been shown to modulate the release of Ca^{2+} from the TC.

FKBP12 is a ubiquitous, soluble 12-kDa protein that is tightly bound to the skeletal muscle RyR, with one FKBP12 per RyR monomer (Jayaraman et al., 1992; Timerman et al., 1993). FKBP12 is a member of a class of proteins that have peptidyl–prolyl *cis–trans* isomerase activity that is inhibited by the binding of the immunosuppressants, FK506 and rapamycin (Harding et al., 1989; Galat, 1993). The clinical immunosuppressive and pharmacological effects of FK506 and rapamycin are mediated by the drug-FKBP12 complexes (Schreiber and Crabtree, 1992). No biological role for intracellular FKBP12, independent of FK506 or rapamycin, had been demonstrated until it was discovered that removal of FKBP12 from skeletal muscle RyR activated Ca^{2+} release from vesicles and activated ryanodine-sensitive Ca^{2+} channels in bilayers (Timerman et al., 1993; Mayrleitner et al., 1994; Ahern et al., 1994; Timerman et al., 1995) and that FKBP12, added to recombinant RyR, strongly modulates the gating of the channel (Brillantes et al., 1994). FKBP12 is also tightly bound to the IP_3 receptor (Cameron et al., 1995) and to the Type 1 cell surface receptor for transforming growth factor β (Wang et al., 1994). The closely related FK506-binding protein, FKBP12.6, is bound to the cardiac isoform of the RyR (Timerman et al., 1994, 1996; Lam et al., 1995; Kaftan et al., 1996). Extracellular FKBP12 has chemotactic activity (Leiva and Lyttle, 1992) and activates neutrophils (Bang et al., 1995).

Incubation of TC vesicles with FK506 or rapamycin removes FKBP12 from the RyR (Timerman et al., 1993, 1995) and causes an increase in Ca^{2+} release via the RyR (Timerman et al., 1993; Brillantes et al., 1994). Skeletal muscle RyR channels stripped of FKBP12 maintain their Ca^{2+}-activation and Mg^{2+}-inhibition properties and sensitivity to ryanodine and ruthenium red (Mayrleitner et al., 1994). However, Mayrleitner et al. found that these stripped channels are more sensitive to activation by caffeine and

Received for publication 22 July 1996 and in final form 29 October 1996.

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0006-3495/97/01/146/17 $2.00

show an increase in open probability (P_o) and mean open times (T_o) at cytoplasmic [Ca^{2+}] between 70 nM and 1.2 μM. Recombinant RyRs, which are FKBP12 deficient, are sensitive to caffeine, Mg^{2+}, ryanodine, ruthenium red, and ATP, but the channels are far less sensitive to Ca^{2+} activation and open predominantly to substates with conductances of ~0.25, ~0.5, and ~0.75 of the maximum conductance (Brillantes et al., 1994). Openings of the recombinant channels to the normal maximum conductance were observed only in 3/32 experiments. When FKBP12 was coexpressed with the RyR, or was added to the bilayer, the recombinant channel opened mostly to the full conductance in only 40% of experiments (Brillantes et al., 1994). The interpretation of these experiments is complicated because of the frequent failure of the recombinant channel to open to the maximum conductance in the presence of FKBP12. The native skeletal RyR opens to the maximum conductance under most conditions. Substates have been reported only rarely in RyRs incorporated into bilayers from TC vesicles (Liu et al., 1989; Ahern et al., 1994). Although substate activity is more common in detergent-purified channels (Lai et al., 1988; Ma et al., 1988; Smith et al., 1988; Hymel et al., 1988; Liu et al., 1989), all these channels open to the maximum conductance. The experiments with the recombinant channels (Brillantes et al., 1994) do show that subconductance activity is greater when FKBP12 is not expressed. However, it is not clear whether many RyRs expressed with FKBP12 fail to open to the maximum conductance because of the Sf9 expression system, because of the detergent purification, or because FKBP12 is not bound.

Our previous studies showed that FK506, added to RyRs that had been incorporated into bilayers from TC vesicles, causes an increase in channel activity at subactivating, activating, and inactivating [Ca^{2+}], with the appearance of a prominent subconductance state at ~0.25 of the maximum conductance (Ahern et al., 1994). RyR channel activity to subconductance states after addition of FK506 has also been observed by Brillantes et al. (1994) and by Ma et al. (1995) but was not reported in the FKBP12-stripped RyR channels examined by Mayrleitner et al. (1994). There are similar conflicting views about the effects of immunosuppressant drugs on cardiac RyRs, with a substantial increase in substate activity seen in one study (Kaftan et al., 1996) but not in a second study (Timerman et al., 1996).

The activation and appearance of subconductance activity in native RyRs exposed to immunosuppressants may have been due to removal of the FKBP from the RyR in the bilayer or to a direct effect of the drug on the RyR. Therefore the aim of the present study was to investigate these possibilities by examining the single-channel properties of RyRs from skeletal muscle TC vesicles that had been depleted of FKBP12. These RyRs, stripped of FKBP12, were modulated, in a way similar to that for native channels, by ATP, ryanodine, ruthenium red, and Ca^{2+} but had increased activity at low [Ca^{2+}] (0.1–5 μM) and high [Ca^{2+}] (0.5–10 mM). The channels showed pronounced subconductance activity to ~0.25, ~0.50, and ~0.75 of the maximum conductance. The three subconductance states together with the maximum open conductance were decreased by the same proportion when the channels were exposed to ryanodine. The results show that the appearance of subconductance levels in the RyR can be attributed to removal of FKBP12, which, therefore, has profound effects on the normal coordinated gating of the native channel. The presence of four conductance levels, at approximately equal multiples of 0.25 of the maximum conductance, suggests that a conducting pathway may be associated with each of the four subunits of the RyR, as previously proposed by Brillantes et al. (1994) and in earlier papers that reported the presence of subconductance activity in purified RyRs (Lai et al., 1988; Liu et al., 1989; Smith et al., 1988). That the relative conductance levels were maintained in the presence of ryanodine indicates that ryanodine alters the conformation of each of the four conducting pathways equally. Some of these results have been published in abstract form (Ahern et al., 1995, 1996).

MATERIALS AND METHODS

Materials

Palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine, and palmitoyl-oleoyl-phosphatidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL). The anti-FKBP12 monoclonal antibody, 3F4-70, was a gift from Fujisawa Pharmaceutical Co. Ltd.(Osaka, Japan), rapamycin was a gift from Wyeth-Ayerst (Princeton, NJ), and ryanodine was obtained from Calbiochem-Novabiochem (San Diego, CA) and Latoxan (Rosans, France). Other chemicals and biochemicals were from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

Isolation of TC membranes and removal of FKBP12

SR vesicles were prepared as described by Ahern et al. (1994) by methods based on Saito et al. (1984). Back and leg muscles were dissected from New Zealand rabbits, snap frozen in liquid N_2, and stored at −70°C. Frozen cubes of muscle were pulverized and homogenized in Buffer A (imidazole, 20 mM; sucrose, 300 mM; adjusted to pH 7.4 with HCl). TC vesicles containing the RyR were collected from the 38–45% (w/w) interface of a discontinuous sucrose gradient after centrifugation. All procedures were performed at 4°C, and buffers contained the following protease inhibitors: leupeptin, 1 μM; pepstatin A, 1 μM; benzamidine, 1 mM; phenylmethylsulphonyl fluoride, 0.7 mM. Protein was assayed by the BioRad DC protein assay based on Lowry et al. (1951), with bovine serum albumin used as a standard.

TC vesicles (2 mg/ml) were incubated with rapamycin (0.05–40 μM) in Buffer A at 37°C for 15 min or at room temperature for 1 h. Solutions for control incubations (without rapamycin) and for incubations with the drug contained 1% ethanol (the solvent for rapamycin) and the protease inhibitors 5 μg/ml leupeptin and 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. Following incubation, membranes were pelleted at 540,000 g for 15 min in a Beckmann TL100 centrifuge, washed once in Buffer A, and resuspended in Buffer A at 2 mg/ml.

Anti-FKBP12 peptide antibodies

The peptide VQVETISPGDGRTFPKC, which corresponds to the *N*-terminal sequence (2–17) of human recombinant FKBP12 (Standaert et al., 1990), with an added C-terminal cysteine, was synthesized by the Austra-

lian National University's Biomolecular Resource Facility. The peptide (5 mg) was coupled to keyhole limpet hemocyanin (5 mg), activated with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester by a method based on Green et al. (1982). New Zealand White rabbits were immunized subcutaneously with 0.8 mg of keyhole limpet hemocyanin–peptide conjugate in Freund's complete adjuvant, followed by booster injections in Freund's incomplete adjuvant, 0.8 and 0.5 mg, at three-week intervals. Preimmune and immune (one week following boosters) sera were collected for testing. Immunoglobulins were isolated from the antisera by caprylic acid treatment and precipitation in 50% ammonium sulfate (Reik et al., 1987), and anti-FKBP12 peptide antibodies were purified by affinity chromatography by use of Pierce (Rockford, IL) immunpore antigen/antibody immobilization kit #2.

Electrophoresis and Western immunoblots

Control and FKBP12-depleted TC proteins were separated on 15% SDS-polyacrylamide gels by use of the buffer system of Laemmli (1970) and transferred to Immobilon-P membranes (Millipore, Boston, MA) in 10 mM 3-[cyclohexylamino]-1-propanesuphonic acid, pH 11.0, 10% ethanol, at 75 V for 30 min, by a Mini Trans-Blot Cell (Biorad, Hercules, CA). Membranes were blocked with 5% skim milk powder in 20 mM Tris, 500 mM NaCl, pH 7.5, incubated with affinity purified antipeptide immunoglobulin (20 μg/ml) or anti-FKBP12 monoclonal antibody 3F4-70 (12 μg/ml) for 2–4 h at room temperature or overnight at 4°C, followed by antirabbit or antimouse IgG horseradish peroxidase conjugate (Silenus Laboratories, Hawthorn, Australia) at a 1:2000 dilution in 1% BSA in 20 mM Tris, 500 mM NaCl, pH 7.5, containing 0.1% Tween and visualized with an enhanced chemiluminescence kit (Amersham, Little Chalfont, Bucks, England). After developing, the films were scanned with a Novaline gel documentation system (Novex, San Diego, CA) and the density of antibody staining analyzed digitally by the Molecular Dynamics (Kew, Australia) ImageQuant software, V. 3.3. Relative amounts of FKBP12 in FKBP12-depleted vesicles were estimated from a standard curve, constructed from the densities measured for FKBP12 in known amounts of native TC vesicles (0.5–10 μg), which were subjected to electrophoresis and blotted from the same gel as the FKBP12-depleted samples. Data from six Western blots of four rapamycin incubations using TC from three vesicle isolations were analyzed.

Artificial lipid bilayers and TC vesicle fusion

The following techniques for lipid bilayers and the measurement and analysis of single-channel activity are described in more detail in Ahern et al. (1994) and Laver et al. (1995).

Lipid bilayers were formed from a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine, and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, w/w), across apertures (diameters 150–300 μm) in the walls of 1.5-ml Delrin cups. TC vesicles were added to the *cis* chamber at a final concentration of 1–10 μg/ml. Ligand sensitivity indicated that the cytoplasmic side of the RyR faced the *cis* chamber. The *cis* solution contained 250 mM CsCl, 10 mM *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, and 1 mM $CaCl_2$, pH 7.5, and the *trans* solution contained 50 mM CsCl, 10 mM *N*-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, and 1 mM $CaCl_2$, pH 7.5. Free [Ca^{2+}] was adjusted by perfusion with solutions containing 2 mM 1,2-bis(2-aminophenoxy)ethane-*N*, *N*, *N*′, *N*′-tetra-acetic acid titrated to the desired free [Ca^{2+}] with $CaCl_2$ by use of a Ca^{2+}-sensitive electrode (ION83 Ionmeter, Radiometer, Copenhagen). Experiments were performed at 20–25°C.

Recording single-channel activity

Voltage was controlled and single-channel activity recorded by means of an Axopatch 200 amplifier (Axon Instruments, Foster City, CA). The *cis* and *trans* chambers were connected to the amplifier head stage by Ag/AgCl electrodes in agar salt bridges. Electrical potentials are given with respect to the *trans* chamber, and positive current is directed from the *cis* to the *trans* solutions. Unless otherwise stated, the bilayer potential was held at +40 mV (near the Cl^- equilibrium potential). Current was monitored on an oscilloscope and recorded at a bandwidth of 5 kHz on videotape by pulse code modulation (Model 200; A. R. Vetter). Current signals were filtered (four-pole Bessel, −3 dB) at 1 or 2 kHz and digitized via a TL-1 DMA interface (Axon Instruments) at 2 or 5 kHz.

Analysis of data

Single-channel activity was analyzed with Channel2 software (developed by M. Smith and P. W. Gage) to yield the following parameters: mean open time (T_o), frequency of opening (F_o), mean closed time (T_o), open probability (P_o, the sum of all open times as a fraction of total time), and mean current (I', the integral of the current divided by total time). Open times were defined as intervals at which the current exceeded a discriminator set above the baseline noise at ~10–~20% of the maximum open level rather than at 50% of the maximum open level, to include openings to subconductance levels. Unless otherwise stated, analysis was performed on ≥90 s of continuous recordings.

Plots of P_o versus [Ca^{2+}] shown in Fig. 10 below were the least-squares fits to a generalized Hill equation described by Laver et al. (1995):

$$P_o = P_{max}(1/(1 + (K_A/[Ca^{2+}])^{H_A})) \cdot (1 - (1/(1 + (K_I/[Ca^{2+}])^{H_I}))), \quad (1)$$

where P_{max} is the maximum open probability of the activated channel, K_A and K_I are the [Ca^{2+}] for half-maximal activation and inhibition, respectively, and H_A and H_I are the Hill coefficients for activation and inhibition, respectively. To fit Eq. 1 we set H_A and H_I at 1.0, approximating values reported in the literature (see Laver et al., 1995).

Current levels were analyzed by mean-variance analysis (Patlak, 1988). A window of 10 data points was moved along the record. The mean current was plotted versus variance pairs as histograms in which the maintained current levels formed low-variance regions. Amplitude histograms were constructed from the bins of the mean-variance histogram that fell below a set level, which excluded high-variance regions associated with transitions. Peaks in the amplitude histogram, corresponding to conductance levels, were fitted by Peakfit software (Jandel) with multiple Gaussian functions.

Analysis of event durations for different conducting levels

Event times of different conductance levels were analyzed by the EVPROC program (D. R. Laver, in Kourie et al., 1996b). EVPROC fits the current signal with an idealized record consisting of putative current transitions, after evaluating the significance of each observed change in the current level. We performed dwell-time analysis for substate and maximum conductances on the idealized record by assigning a current range or "window" for each level (estimated from amplitude histograms). A transition into the window was recorded as an opening and a transition out of the window as a closure. In Fig. 11 below, analysis of event times was confined to those in the lowest substate level, S1, and those in the maximum open state, O. A window was assigned for the substate level (openings mostly to S1) and another for the maximum open state (openings mostly to O), indicated by the dotted lines in Fig. 11 *A* and *C*. Dwell-time distributions were plotted by the log-bin method (Sigworth and Sine, 1987), and least-squares fits of a multiple exponential function were obtained.

Statistical analysis

Differences between mean values were evaluated by Student's *t*-test. Significance was set at the 0.05 level. For comparison of two different groups of channels, a two-sample, two-tailed *t*-test was used.

RESULTS

Removal of FKBP12 from TC vesicles

In most experiments FKBP12 was removed from TC vesicles by incubation with rapamycin (0–30 μM) at 37°C for 15 min, essentially as described by Timerman et al. (1993, 1995). The amount of FKBP12 remaining associated with TC vesicles was visualized on Western blots of SR proteins immunostained with affinity-purified antipeptide antibodies (Fig. 1 *A*). The immunostaining of the ~12-kDa band (*arrows*) was specific for the *N*-terminal peptide sequence of FKBP12 because preincubation of the antibodies with the immunizing peptide specifically inhibited the immunostaining of the ~12-kDa band (results not shown). In contrast, the identities of immunostained bands at ~30 kDa and >50 kDa are unknown. The latter two bands were not depleted by incubation of the SR vesicles with rapamycin (Fig. 1 *A*) or observed with secondary antibody alone but were also detected by antibodies in the immune serum that were not retained on the peptide affinity column during purification. To confirm that the 12-kDa band was FKBP12, we stripped the Western blot in Fig. 1 *A* and reprobed it with an anti-hFKBP12 monoclonal antibody, 3F4-70. Only the 12-kDa band was immunostained by the monoclonal antibody (Fig. 1 *B*). As the intensity of immunostaining was less than that for the antipeptide antibody, the monoclonal antibody was not used routinely for the detection of FKBP12.

The percentage of FKBP12 remaining in TC vesicles after incubation with rapamycin is shown in Fig. 1 *C*. The solid curve is the best least-squares fit of a Hill equation to the data. The K_D and the Hill coefficient H were 0.33 μM and 1, respectively. The K_D for rapamycin to remove FKBP12 is similar to that obtained for removal of FKBP12 by FK506 (~0.12–~0.5 μM; Timerman et al., 1993) and is very close to the K_D for [^{35}S]–FKBP12 binding to stripped TC vesicles (0.33 ± 0.16 μM; Timerman et al., 1995). The data points show that zero density was observed at 3 μM rapamycin, whereas the Hill equation predicts that ~10% FKBP12 will remain at 3 μM rapamycin. Hence 10% of the FKBP12 content of the TC vesicles represents the limit of detection under the conditions used for immunostaining.

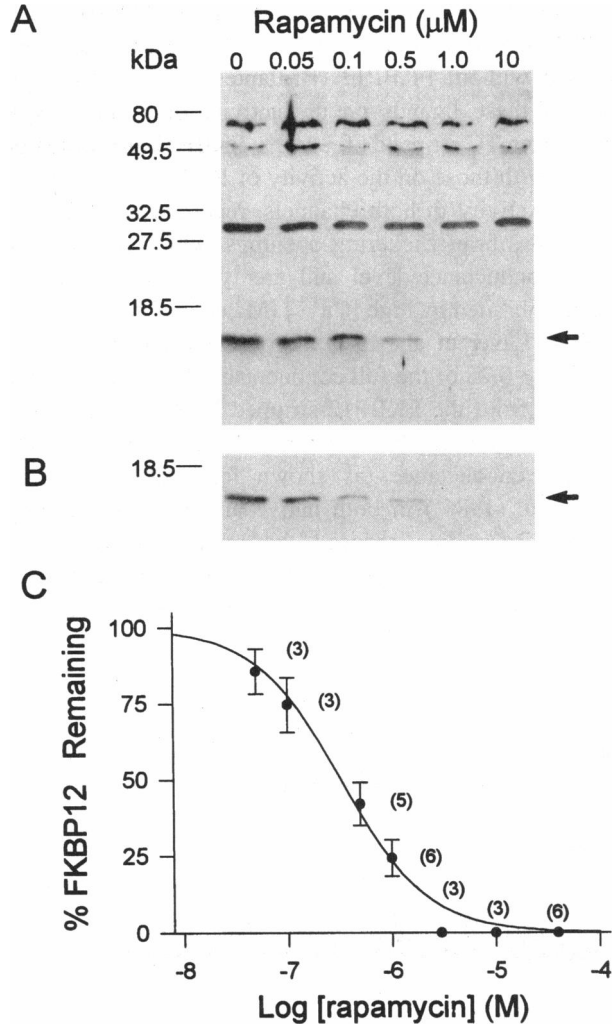

FIGURE 1 Removal of FKBP12 from TC vesicles by incubation with rapamycin for 15 min at 37°C. *A*, Western blot of TC proteins immunostained with the anti-FKBP12-peptide antibody as described in Methods. The arrows shows the positions of the FKBP12: the intensity of this band decreases with increasing rapamycin concentrations. *B*, The Western blot in *A* was "stripped" as described in the enhanced chemiluminescence kit (Amersham) and reprobed with the anti-FKBP12 monoclonal antibody (3F4). The only band immunostained was the ~12-kDa band, whose density once again decreased with increasing [rapamycin]. *C*, The percentage of FKBP12 remaining in TC vesicles after incubation with rapamycin was estimated (Methods) and plotted against [rapamycin]. The numbers of Western blots from which average data were obtained are given in parentheses, and the vertical bars show ±1 SE. Data were fitted with a modified Hill equation:

$$\%\ \text{FKBP12 Remaining} = 100(1 - (1/(1 + K_D/[\text{rapamycin}]^H))).$$

The best least-squares fit yielded a half-maximal concentration for FKBP12 dissociation, K_D = 0.33 μM, and a Hill coefficient, H = 1.

Single-channel properties of RyRs with FKBP12 removed

Considerable variability was observed among RyRs obtained from individual preparations of FKBP12-depleted TC vesicles. However, all RyR channels from FKBP12-depleted TC showed subconductance activity throughout their records. Most single-channel data were obtained from TC vesicles that were stripped of ~100% of FKBP12 (i.e., incubated with 10–30 μM rapamycin). Measurements were made on 60 channels from 7 rapamycin incubations of TC vesicles from 5 different SR preparations.

FKBP12-depleted RyRs respond to ATP, ruthenium red, and ryanodine

Removal of FKBP12 did not affect the ability of the RyR to respond to several ligands, including ATP, ruthenium red,

and ryanodine, as reported for skeletal RyR (Mayrleitner et al., 1994; Timerman et al., 1995) and for recombinant RyR expressed without FKBP12 (Brillantes et al., 1994). The effects of these ligands on the activity of a single RyR channel from TC stripped of ~100% FKBP12 are compared in Fig. 2 with those on the activity of RyR from native TC vesicles. Activity in both channels, recorded in 1 mM *cis* Ca^{2+}, shows brief flickering openings, which rarely reach the full conductance level and are typical of activity in channels inhibited by high [Ca^{2+}] (Ma et al., 1988; Ahern et al., 1994; Laver et al., 1995). Prominent subconductance activity at ~0.25 of the full conductance (S1) is apparent in the record from the FKBP12-stripped RyR. Addition of 1 mM ATP increased the number of long openings to the maximum conductance (as shown for skeletal RyR by Smith et al. (1986)) in both native and FKBP12-stripped channels. Ryanodine reduced the main conductance to ~50% of that recorded before the addition of the drug in both native and FKBP12-stripped channels and also reduced S1 to ~50% in the FKBP12-stripped channel. The actions of ryanodine are considered in more detail in a later section. Ruthenium red inhibited RyR activity in both cases. In native RyRs, ATP activation was observed in 13/13 channels, ryanodine modification in 8/9 channels, and ruthenium red inhibition in 5/5 channels. In FKBP-stripped channels, ATP activation was observed in 10/10 channels, ryanodine modification in 17/21 channels, and ruthenium red block in 8/9 channels. (The concentration of ruthenium red indicated in Fig. 2 (80 μM) was based on weight and was much higher than the 1 μM used by Smith et al. (1988) to block the native RyR. The concentration estimated from its spectrum was ~30 μM, i.e., the concentration quoted by others (Lai et al., 1988; Hymel et al., 1988). Although we did not study this effect in detail, it appeared that the FKBP12-stripped RyRs that had been modified by ryanodine required a higher concentration of ruthenium red than did native channels to ensure inhibition. Brillantes et al. (1994) used 20 μM ruthenium red to block the recombinant RyR channel.)

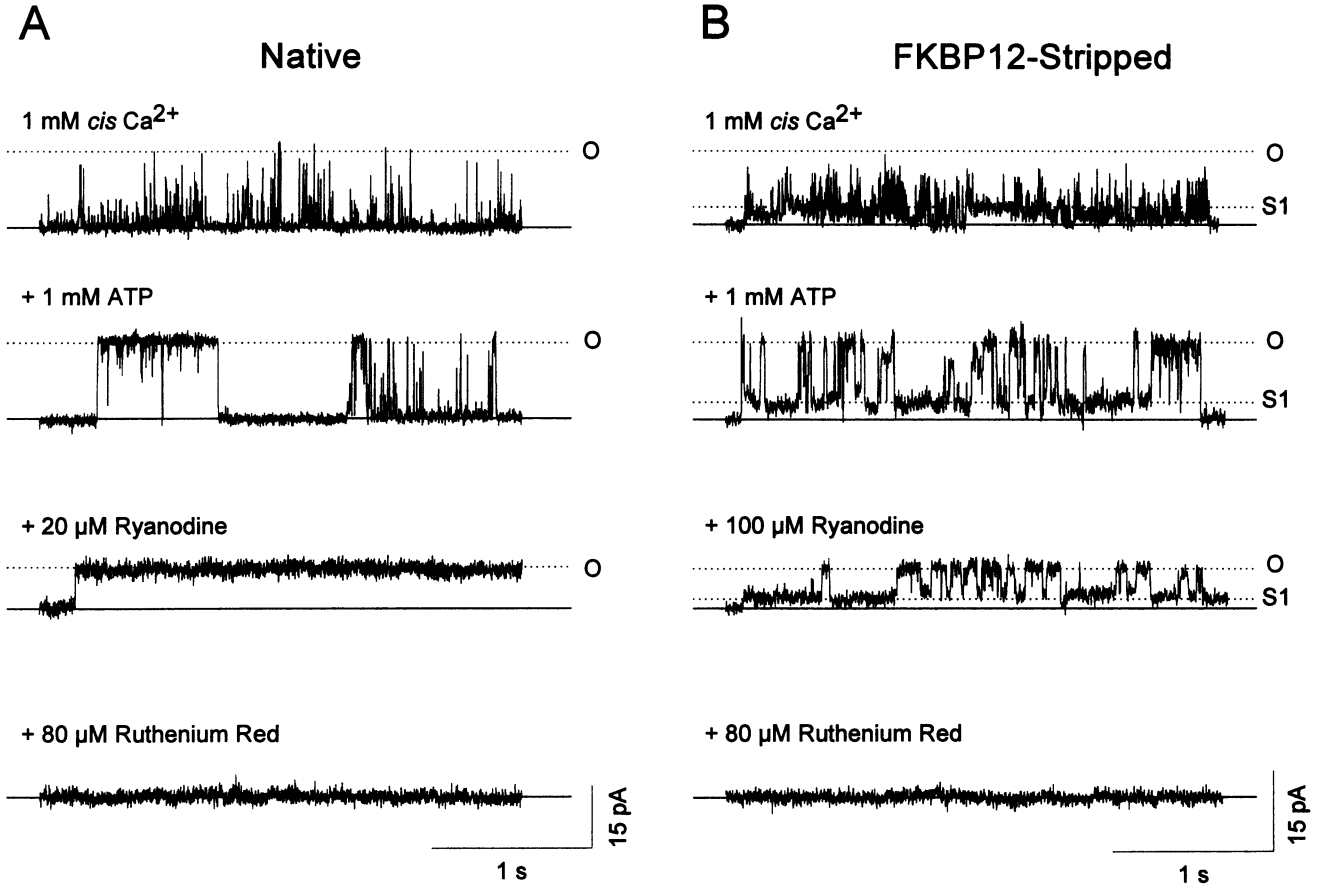

FIGURE 2 RyR channels, stripped of FKBP12, respond to the same ligands as native RyRs. Representative channel activity from single RyRs incorporated into bilayers from native TC (*A*) and from ~100% FKBP12-stripped vesicles (*B*). Bilayer potential was +40 mV, and solutions contained 250/50 mM CsCl and 1/1 mM Ca^{2+} (*cis/trans*). The native and stripped channels opened to a maximum open level (O). In addition, the stripped channel opened to a subconductance level (S1). Both native and stripped channels show low levels of activity in 1 mM *cis* Ca^{2+} (*topmost panels*), are activated by the addition of ATP (*second panels*), are forced into a reduced maximum conductance by ryanodine (*third panels*), and are inhibited fully by ruthenium red (*fourth panels*).

FKBP12 depletion does not alter the maximum RyR conductance

The maximum conductance of RyR channels was not affected by the removal of FKBP12, as found by Mayrleitner et al. (1994) and by Brillantes et al. (1994) for recombinant RyR with and without FKBP12. The maximum conductances, measured at +40 mV, were 225 ± 6 pS (n = 8) for control channels and 237 ± 4 pS (n = 32) for FKBP12-depleted channels. The current–voltage relationship measured in some of the channels gave a reversal potential of ~−20 mV in both control and FKBP12-stripped channels (see Fig. 4 *C*). The conductance of ~230 pS is low compared with higher values of ~500 pS reported by others (Ma et al., 1995; Sitsapesan and Williams, 1995). This is due to the 1 mM Ca^{2+} in the *trans* solution, which competes with Cs^+ for binding sites in the channel pore and reduces the Cs^+ current (Tinker et al., 1992). The channels have a conductance of ~480 pS when *trans* [Ca^{2+}] is 1 μM (Ahern et al., 1994).

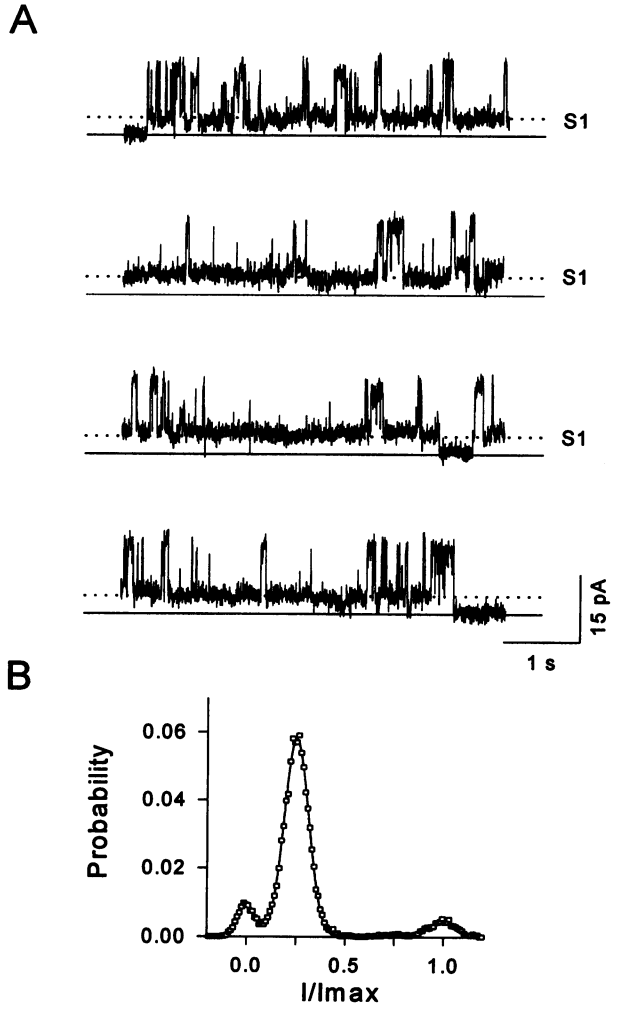

FIGURE 3 Subconductance channel activity in a RyR incorporated into planar bilayers from TC vesicles stripped of ~100% of their FKBP12. *A*, 20-s segment of continuous activity at +40 mV, with 1 mM Ca^{2+} and 1 mM ATP in the *cis* solution. Prominent activity at the subconductance level, S1, is shown in the records (*broken line*). *B*, An amplitude histogram constructed from mean-variance analysis of the 20 s of data from the above channel. Current was expressed relative to the maximum current (I/I_{max}), collected into bins of 0.01 and fitted by multiple Gaussian functions as described in Methods. The curve shows the relatively high probability of the 0.25 conductance level (S1) for this channel.

FKBP12 removal increases RyR activity to submaximal conductance levels

The most prominent effect of FKBP12 removal on RyR channels was a very marked increase in the number of openings to subconductance levels. Substate activity was observed throughout the recordings from all FKBP12-depleted channels (n = 60, 25–100% FKBP12 removed) but was seen only occasionally in 4 of 14 control-incubated channels and in 23 of 107 native RyR channels. Substate activity in a single RyR channel from FKBP12-stripped TC is shown in Fig. 3 *A*. The record of 20-s continuous single-channel activity was obtained with 1 mM *cis* Ca^{2+} and 1 mM ATP. Channel activity was dominated by long openings (>200 mS) to a low conductance level. The histogram in Fig. 3 *B*, obtained by mean-variance analysis of the record shown in Fig. 3 *A*, demonstrates the high probability of activity at 0.25 of the maximum conductance (S1).

Shorter records of a 25% FKBP12-depleted RyR, selected to illustrate maintained channel activity at the subconductance levels S1, S2, and S3, are shown in Fig. 4 *A*. Mean-variance analysis of the selected channel openings gave five clearly defined peaks, one corresponding to the closed state, one to the fully open state, and three that were associated with substate activity. The substates had fractional conductances of 0.21 (S1), 0.41 (S2), and 0.81 (S3) of the maximum conductance (Fig. 4 *B*). The current–voltage relationships for the maximum conductance and for S1, S2, and S3 are shown in Fig. 4 *C*. The curves for S1, S3, and O reversed close to −20 mV (there were insufficient data to permit us to fit a curve to S2). The similar reversal potentials show that the permeability of Cs^+ relative to that of Cl^- is the same for the maximum open state and for each of the substates.

The fractional conductances of the substates were essentially the same at membrane potentials in the range −80 to +40 mV. For one channel, incorporated from ~25%-depleted TC and activated by 1 μM *cis* Ca^{2+}, mean-variance analysis was performed on 7 s of continuous channel activity at +40 mV and on 1.5 s at −60 mV. At +40 mV, two substates were detected, S1 and S3, with fractional conductances of 0.20 and 0.81, respectively. At −60 mV, all three substate levels were observed, with fractional conductances of 0.28 (S1), 0.45 (S2), and 0.83 (S3)

Conductance levels at +40 mV were analyzed by mean-variance analysis of selected records of 39 RyRs from FKBP12-depleted TC. The frequency distribution of the conductance levels shows three peaks with fractional conductances of (mean ± SE) 0.25 ± 0.02 (S1), 0.50 ± 0.02 (S2), and 0.77 ± 0.05 (S3) (Fig. 5 *A*). The RyRs used to

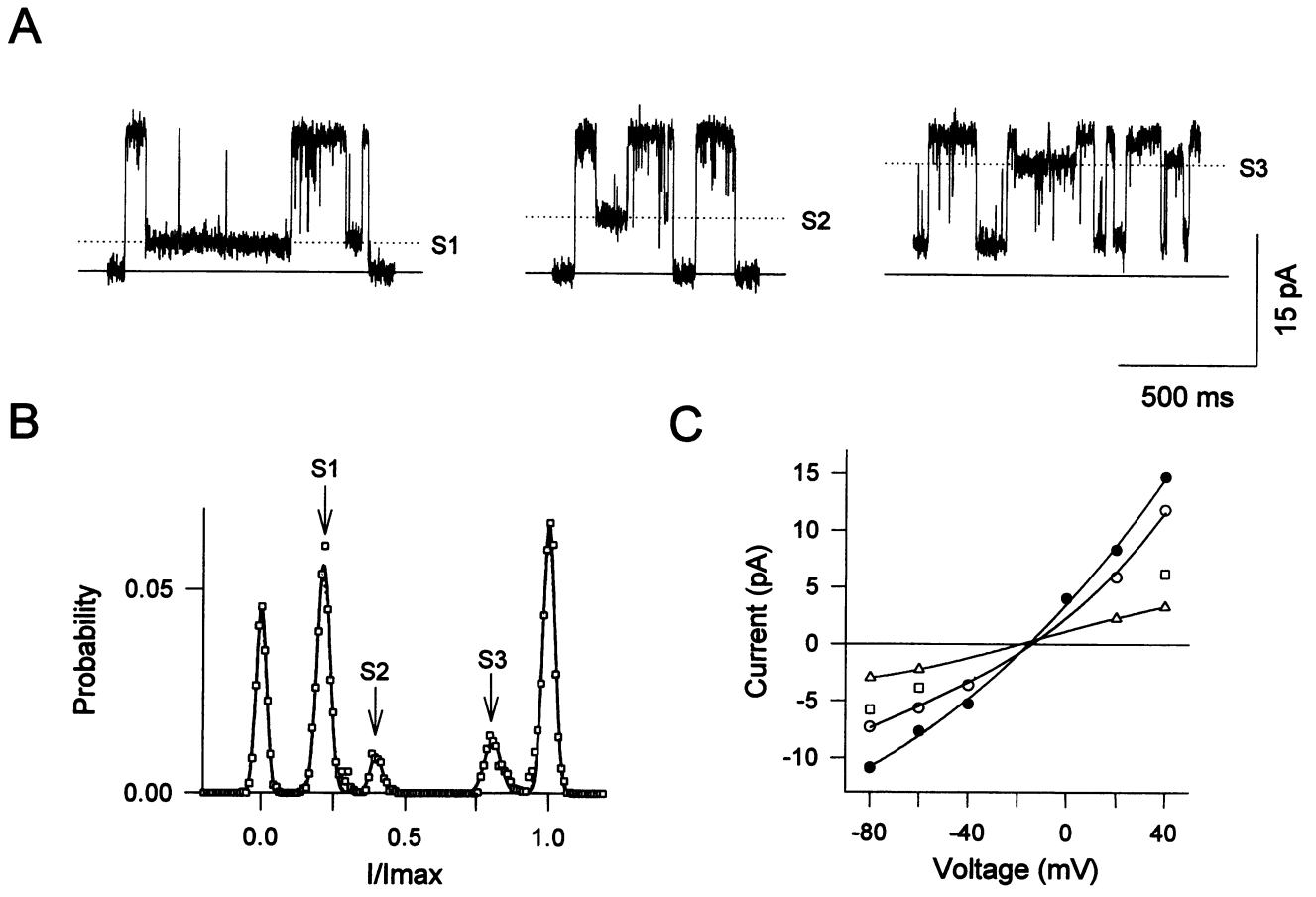

FIGURE 4 Channel activity to subconductance levels is observed in RyRs incorporated from TC vesicles depleted of ~25% of their FKBP12. *A*, Selected segments of channel activity showing openings to the S1, S2, and S3 subconductance levels. Bilayer potential was +40 mV, and the channel was activated by 10 μM *cis* Ca^{2+}. *B*, Amplitude histogram constructed from mean-variance analysis of the selected data in *A* (see the legend of Fig. 3) showing the probability of the subconductance levels S1, S2, and S3. *C*, Current–voltage relationship for the maximum open level, O (●) and the three subconductance levels, S1 (Δ), S2 (□), and S3 (○) for the channel shown in *A*. The solid curves are the best fits to a third-order polynomial function. The currents all reversed close to −18 mV, and the slope conductances calculated at this potential were 60 pS (S1), 160 pS (S3), and 211 pS (O).

construct the histogram included channels from TC vesicles that were depleted of 25–100% of their FKBP12 and channels exposed to a range of *cis* [Ca^{2+}] from 1 μM to 1 mM, and in the presence or absence of 1 mM ATP. The asymmetry and widths of the peaks in the frequency distribution could reflect variability among the single channels, the conditions used to activate the channels, or channel activity to additional subconductance levels. More than three substates were clearly observed in some channels (data not shown) and would contribute to peak width and asymmetry. No consistent effects of the extent of FKBP12 removal, or of the activating ligand, on the relative conductance levels were observed. Furthermore, the prominent clustering of current levels into three peaks in Fig. 5 suggests that the relative levels were largely independent of the experimental conditions. Note that, as short records with distinct subconductance activity were selected to construct the histogram, the frequency of each substate in Fig. 5 does not reflect the relative frequency observed when long segments of data were analyzed (see, e.g., Fig. 3 *B*).

Subconductance levels in native RyR channels

Subconductance levels, with fractional conductances similar to those seen in FKBP12-depleted channels, were observed infrequently in 20% of RyR channels incorporated from native and control-incubated TC vesicles. Occasional subconductance activity in native RyRs was observed previously (Liu et al., 1989). These subconductance states were not usually prominent in histograms when continuous records longer than 10 s were analyzed. The frequency distributions of substates from selected records of 15 native RyR channels (Fig. 5 *B*) show that fractional conductances clustered in the ranges 0.20–0.30, 0.40–0.55, and 0.70–0.85, with the most frequent level being ~0.25 of the maximum level. Although there was considerable variability among channels, these three substate levels share the same relative conductances with those observed far more frequently after removal of FKBP12. The substate levels in native and control-incubated RyRs had the same I-V relationships as FKBP12-depleted RyRs, with the same reversal

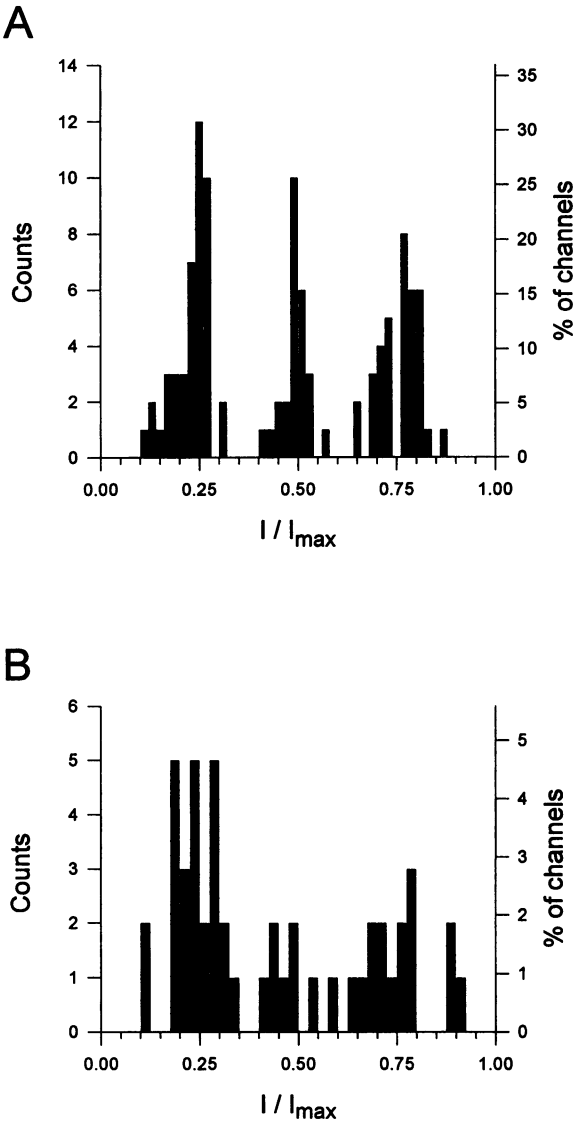

FIGURE 5 Substate distributions in RyR channels incorporated into bilayers from TC vesicles depleted of FKBP12 (*A*) and from native TC vesicles (*B*). Data containing openings to submaximal conductance levels were selected for analysis. *A*, Frequency distribution of substate levels in segments of RyR channel activity (n = 39) from TC vesicles depleted of 25–100% of their FKBP12. Data were included from channels with *cis* [Ca^{2+}] between 1 μM and 1 mM and in the presence or the absence of 1 mM ATP. Current amplitudes were expressed as a fraction of the maximum current and collected into bins of 0.02-pA intervals. *B*, Frequency distribution of substate levels observed infrequently in RyR channels (n = 17) from native TC vesicles. Values were expressed as a fraction of the maximum current and collected into bins of 0.025-pA intervals. Note the different y axes on distributions in *A* and *B*, reflecting the infrequent occurrence of substates in native channels.

potentials (~−20 mV), as the maximum conductance (data not shown).

FKBP12 depletion does not alter the effect of ryanodine on the maximum conductance

Addition of ryanodine (4–100 μM) to the *cis* chamber induced the same slow kinetics and reduced conductance levels in native (8/9), control-incubated (2/2), and FKBP12-depleted RyR channels (17/21) (see, e.g. Fig. 2). Removal of FKBP12 from RyRs did not alter the ryanodine-induced reduction in the maximum conductance at +40 mV. The average conductance was to 0.51 ± 0.01 of that before addition of ryanodine in FKBP12-depleted RyRs (n = 15), and an identical value of 0.51 ± 0.01 was obtained for native and control-incubated RyR channels (n = 9).

The time taken for ryanodine to affect the gating kinetics of the RyR channel was not altered significantly by the removal of FKBP12. Ryanodine increased P_o and reduced the conductance levels within 2–3 min of addition to the bilayer in 17/21 FKBP12-depleted channels. The average latency of induction of the ryanodine gating mode was not significantly different in native and control-incubated RyR channels (1.1 ± 0.4 min, n = 8), in FKBP12-depleted RyR (1.2 ± 0.3 min, n = 16), or in native RyRs that had been exposed to *cis* FK506 or rapamycin in the bilayer (1.5 ± 0.4 min, n = 6).

Ryanodine modification of the subconductance levels in FKBP12- depleted RyR channels

Micromolar ryanodine usually produced a solitary open level in control RyR channels (8/9). In marked contrast, multiple levels were seen in all FKBP12-stripped channels in which activity was modified by ryanodine (n = 17). The relative amplitudes of the submaximal conductance levels in FKBP12-depleted channels were not altered after addition of ryanodine. Examples of channel activity to the S1, S2, and S3 subconductance levels in one FKBP12-stripped channel before and after addition of ryanodine are illustrated in Fig. 6 *A* and *C*. Mean-variance analysis of the data before ryanodine was added (Fig. 6 *B*) yielded three submaximal peaks, at 3.3 pA (S1), 6.2 pA (S2), and 11.4 pA (S3), as well as the maximum peak at 14.9 pA (O). Ryanodine reduced the conductance of the three submaximal peaks to 2 pA (S1), 3.9 pA (S2), and 5.6 pA (S3) and reduced the maximum level to 8.3 pA (O) (Fig. 6 *D*). Each conductance level was reduced to ~50-60% of the level before ryanodine modification.

The effects of ryanodine on FKBP12-depleted channels were independent of the amount of FKBP12 removed (25–100%) or the conditions used to activate the channels before addition of ryanodine (10 μM–1 mM *cis* Ca^{2+}, presence or absence of 1 mM ATP). The histogram in Fig. 7 compares relative conductance levels in 11 channels in the presence of ryanodine (*white bars*) with the substate distribution for control FKBP12-depleted channels (*black bars*) already shown in Fig. 5 *A*. Submaximal peaks in the histogram for ryanodine-modified FKBP12-depleted channels at 0.26 ± 0.02 (S1), 0.48 ± 0.01 (S2), and 0.75 ± 0.02 (S3) of the maximum conductance are comparable with the peaks in the unmodified FKBP12-depleted channels (see the description of Fig. 5). Thus the conductances of the four predominant open states in FKBP12-depleted RyR channels were modified in the same way by ryanodine.

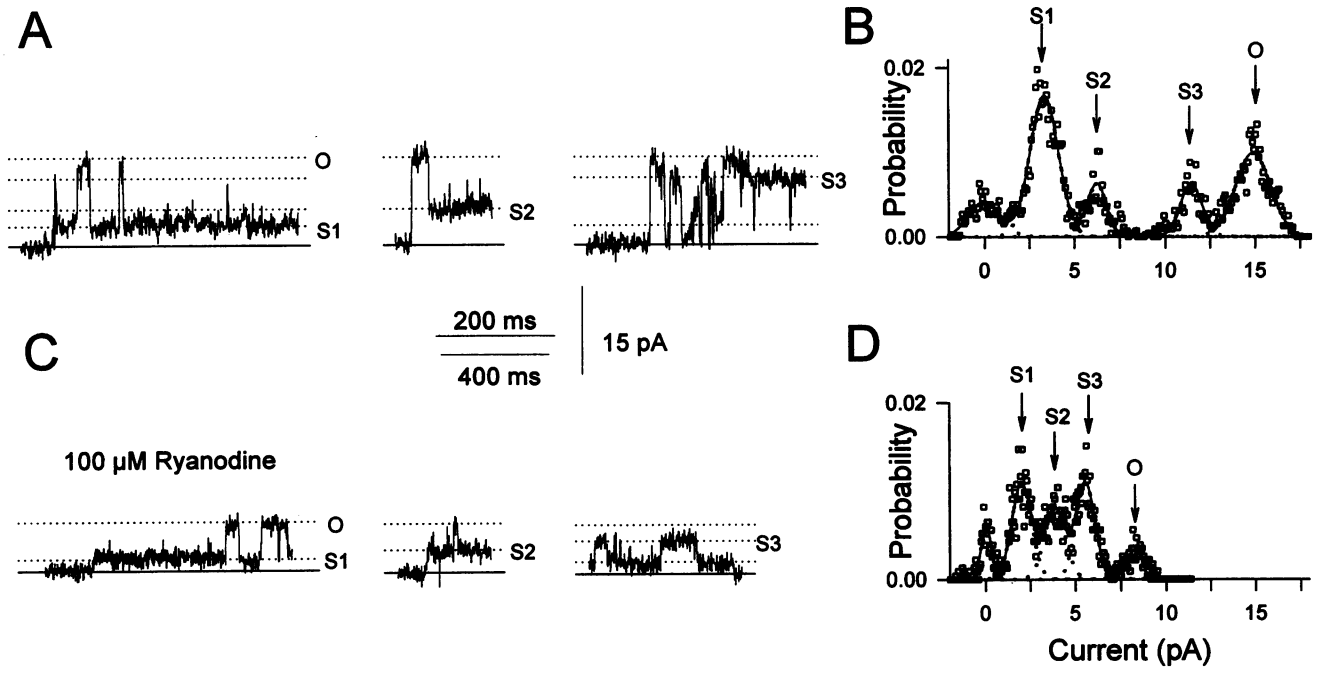

FIGURE 6 Effect of ryanodine on the multiple subconductance levels in a RyR channel, incorporated from TC vesicles stripped of ~100% of FKBP12, recorded at +40 mV, with *cis* 1 mM Ca^{2+} and 1 mM ATP. *A*, Selected activity from one FKBP12-stripped channel before addition of ryanodine, showing the S1, S2, and S3 subconductance levels and the maximum open level, O. *B*, Amplitude histogram constructed by mean-variance analysis of 3 s of selected data. The best fit of a multiple Gaussian function showed peaks at 3.3 (S1), 6.2 (S2), 11.4 (S3), and 14.9 pA (O). *C*, Selected activity of the same channel after addition of ryanodine (100 μM). Note the different time scales for *A* and *C*. *D*, Amplitude histogram constructed by mean-variance analysis of 4 s of selected data after ryanodine addition. The best multiple Gaussian fit showed peaks at 2.0 (S1), 3.9 (S2), 5.6 (S3), and 8.3 pA (O). The conductance of the substate levels (S1–S3) and the maximum open level (O) were all reduced by approximately the same proportion in the presence of ryanodine.

Micromolar ryanodine irreversibly locks native RyRs into the reduced-conductance, high-P_o mode (Smith et al., 1988; Ma et al., 1988), and the conductance and the P_o of most FKBP12-depleted channels were also irreversibly changed. However, two of the FKBP12-depleted channels underwent a transient gating shift, into and out of the ryanodine-modified mode. This transient reversal of the ryanodine effect was never observed in native or control-incubated channels. Records obtained during these shifts in one of the FKBP12-depleted channels are shown in Fig. 8. The channel entered the reduced-conductance, high-P_o mode 30 s after the addition of 7 μM ryanodine (Fig. 8 *A*). The channel returned to the full conductance mode 1 min later (Fig. 8 *B*) and, after a further 15 s, returned to the ryanodine-modified mode (Fig. 8 *C*), which was maintained for the remaining lifetime of the bilayer.

FKBP12-depleted RyRs are sensitive to cis [Ca^{2+}]

We measured the Ca^{2+} dependence of channel activity to characterize further the effects of FKBP-12 depletion on the RyR. In Fig. 9 the single-channel activities, at four different values of *cis* [Ca^{2+}], of control-incubated RyRs are compared with RyR channels from TC vesicles stripped of 75–100% of their FKBP12. Although the FKBP12-depleted channels were activated when *cis* [Ca^{2+}] was increased above 0.1 μM and inhibited by 1–5 mM Ca^{2+}, the FKBP12-depleted channels were more open than the control channels at 0.1 μM Ca^{2+} and 1–5 mM Ca^{2+}. In contrast, the P_o for the FKBP12-depleted channel was not noticeably different from that of the control channel at 10 μM Ca^{2+}. Note that much of the activity in the FKBP12-depleted channels was to subconductance states.

Effects of cis [Ca^{2+}] on P_o in normal and FKBP12-depleted channels

The procedure used for FKBP12 removal did not significantly alter the Ca^{2+} sensitivity of the control-incubated RyR. The Ca^{2+} dependence of P_o for RyR channels from control-incubated TC vesicles was very similar to that of untreated native vesicles (Fig. 10 *A*). In marked contrast, FKBP12-depleted RyRs demonstrated a much broader Ca^{2+} dependence than the control and the native channels. Average data from the 33 FKBP12-depeleted channels are compared in Fig. 10 *B* with average data from the native-plus-control-incubated channels (n = 81). Although the standard errors on the data from FKBP12-depleted channels are much higher than those from control channels, all mean P_o values for the depleted channels were higher than those in control and native channels at subactivating (0.1–1 μM) and inactivating (>100 μM) Ca^{2+} concentrations. A very similar broadening of the relationship between mean current (I') and [Ca^{2+}] was obtained (data not shown). Therefore

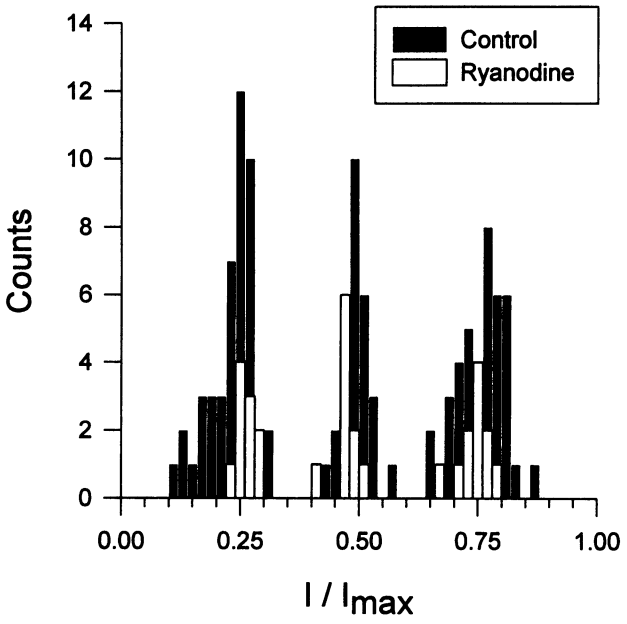

FIGURE 7 Distribution of subconductance levels for the ryanodine-modified, FKBP12-depleted RyR channels. Histograms obtained from selected channel activity of ryanodine-modified RyRs incorporated from FKBP12-depleted TC (n = 11; *white bars*) are compared with those of unmodified, FKBP12-depleted channels (n = 39; *black bars*; data from Fig. 5 *A*). Peaks in the distribution (mean ± SE) of 0.26 ± 0.02, 0.48 ± 0.02, and 0.75 ± 0.02 for the ryanodine-treated channels are very similar to those obtained for unmodified, FKBP12-depleted channels (see the legend of Fig. 5 *A*).

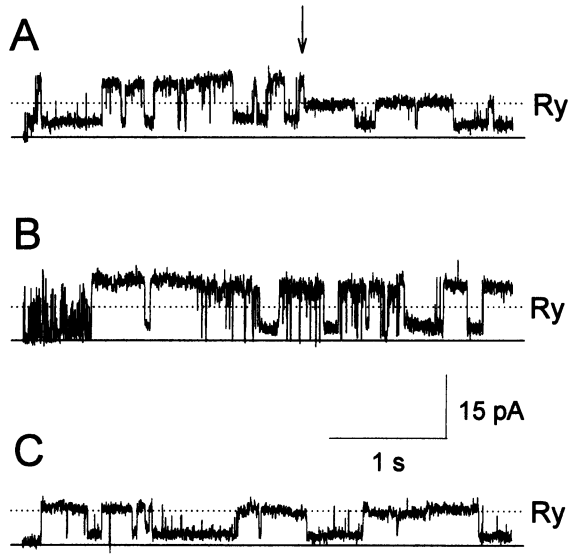

FIGURE 8 Transient reversal of ryanodine modification of a single RyR channel incorporated into bilayers from TC vesicles stripped of ~100% of FKBP12. Current traces measured at +40 mV with 1 μM *cis* Ca^{2+}. *A*, The channel switched into the reduced conductance (*arrow*) of the ryanodine-modified state, (Ry), 30 s after the addition of 7 μM ryanodine. *B*, After the channel reverted to the normal, FKBP-stripped mode, 1 min later. *C*, After the channel returned to the ryanodine-induced conductance levels, 15 s later. The channel remained in the ryanodine-modified state for the remaining lifetime of the bilayer.

the effect of FKBP12-depletion on the Ca^{2+} dependence of P_o was not distorted by the exclusion of submaximal openings that might have been too close to baseline to be detected as clear openings (see below).

The modified Hill curve drawn through the data (see Methods) indicates that there was an ~10-fold increase in the Ca^{2+} sensitivity of channel activation and a 10-fold decrease in the sensitivity of Ca^{2+} inhibition following the removal of FKBP12. However, the maximum P_o of ~0.15 was not altered by FKBP12 removal. A similar shift in the Ca^{2+} activation curve to lower [Ca^{2+}] in FKBP12-stripped channels was reported by Mayrleitner et al. (1994).

Effects of cis [Ca^{2+}] on F_o, T_o, and T_c in normal and FKBP12-depleted channels

A routine analysis of open and closed durations was not performed on all channels because of the increase in activity to low levels of ≤25% of the maximum conductance following removal of FKBP12. It was difficult in some channels to set the discriminator so that all baseline noise was excluded and the full length of channel openings to low conductance levels recorded without artifactual interruption by noise. The open and closed times could be measured unambiguously in approximately half of the FKBP12-depleted channels where records showed low noise and clean transitions. Table 1 compares average frequency of opening (F_o), mean open times (T_o), and mean closed times (T_c) of the FKBP12-depleted channels with parameters measured from control-incubated channels. Data are shown for *cis* Ca^{2+} concentrations of 0.1, 10, and 1 mM. F_o was ~8-fold greater in FKBP12-depleted channels than in control channels at 0.1 μM Ca^{2+}; T_c was reduced ~8-fold, whereas T_o was not significantly different from control. Thus the higher P_o in FKBP12-depleted channels at 0.1 μM *cis* Ca^{2+} was due primarily to an increased frequency of openings rather than to an increase in open times. At 10 μM *cis* Ca^{2+} there was no significant difference in any of the parameters, although T_c and T_o tended to be shorter in the depleted channels and F_o tended to be higher. This result was consistent with those for the similar P_o measured in control and FKBP12-depleted channels at 10 μM Ca^{2+} (see Fig. 10 *B*). In contrast, at inactivating 1 mM *cis* Ca^{2+} the F_o for the FKBP12-depleted channels was ~4-fold higher, and T_o was ~2.5-fold higher, than in control channels. Thus the higher P_o in FKPB12-depleted channels at 1 mM Ca^{2+} may be attributed to the combined effects of the increases in F_o and T_o.

Ligands have differential effects on the proportion of channel activity to the maximum conductance and to subconductance levels

It was apparent that different ligands altered the probability of channel activity to the maximum conductance and to the subconductance levels in different ways. This is illustrated

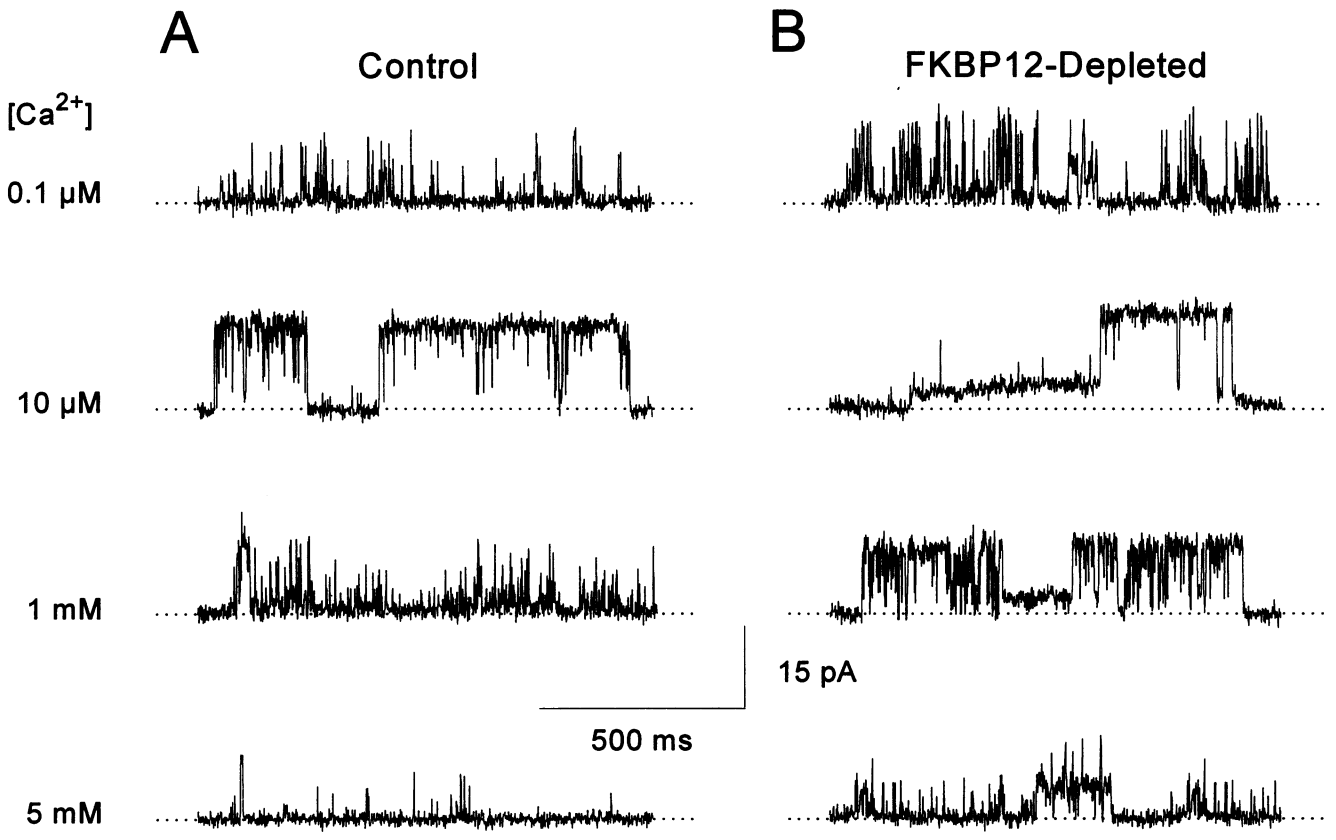

FIGURE 9 Dependence of RyR channel activity on *cis* Ca^{2+}. Representative bursts of activity in current recordings of RyRs from control-incubated TC (*A*) and from TC vesicles stripped of 75–100% of their FKBP12 (*B*), at a bilayer potential of +40 mV. Data are shown for *cis* [Ca^{2+}] of 0.1 μM (*top panels*), 10 μM (*second panels*), 1 mM (*third panels*), and 5 mM (*fourth panels*). *cis* Ca^{2+} was adjusted by perfusion with Ca^{2+}-buffered solutions (see Methods).

by the effects of *cis* [Ca^{2+}] and ATP on FKBP12-depleted channels.

Ca^{2+} inhibition Many records from FKBP12-depleted channels showed longer openings to the S1 level than to the maximum conductance, and this was enhanced when the channel was partially inhibited by 1 mM cis Ca^{2+}. A quantitative measure of enhanced activity at S1 with inhibiting *cis* Ca^{2+} was obtained for a FKBP12-stripped channel in which activity was measured at both 1 μM (Fig. 11 *A*) and 1 mM (Fig. 11 *C*) *cis* Ca^{2+}. The duration of events at S1 and the maximum conductance were measured and compiled by the EVPROC program (see Methods). Open time histograms were plotted by the use of logged bins (Sigworth and Sine, 1987), which display the distribution of open times that ranged from ~1 to 500 ms (Fig. 11 *B* and *D*). The peaks in the histogram correspond to the time constant of each exponential component. With 1 μM *cis* Ca^{2+} the distribution for the maximum conductance is similar to that of the S1 level (Fig. 10 *B*). Both distributions were fitted by the sum of three exponentials with similar time constants. When *cis* Ca^{2+} was increased to 1 mM, the open durations of the maximum conductance were reduced, and the distribution could be adequately fitted by a double exponential. In contrast, the open time distribution for S1 was minimally affected by the increase in *cis* [Ca^{2+}] and, as with 1 μM *cis* Ca^{2+}, was once more fitted by three exponentials. However, there were fewer events associated with τ_3 at 1 mM *cis* Ca^{2+} than at 1 μM Ca^{2+}. The mean open time for the maximum conductance in this channel decreased from 9.3 to 1.2 ms (~8-fold decrease) when *cis* Ca^{2+} was raised from 1 μM to 1 mM, whereas the mean open time for the S1 substate decreased from 15.6 to 4.1 ms (~4-fold decrease). Thus the duration of openings to the S1 substate in FKBP12-depleted RyR was less reduced by millimolar Ca^{2+} than was the duration of openings to the maximum conductance.

ATP activation When ATP was added to FKBP12-stripped RyR channels that were inhibited by 1 mM cis Ca^{2+} the duration of channel openings to the maximum conductance increased markedly, with only a modest increase in the duration of openings to the S1 level (see, e.g. Fig. 2 *B*, top two traces).

DISCUSSION

The subconductance levels and single-channel properties of RyRs incorporated into bilayers from native TC vesicles depleted of known amounts of FKBP12 have been exam-

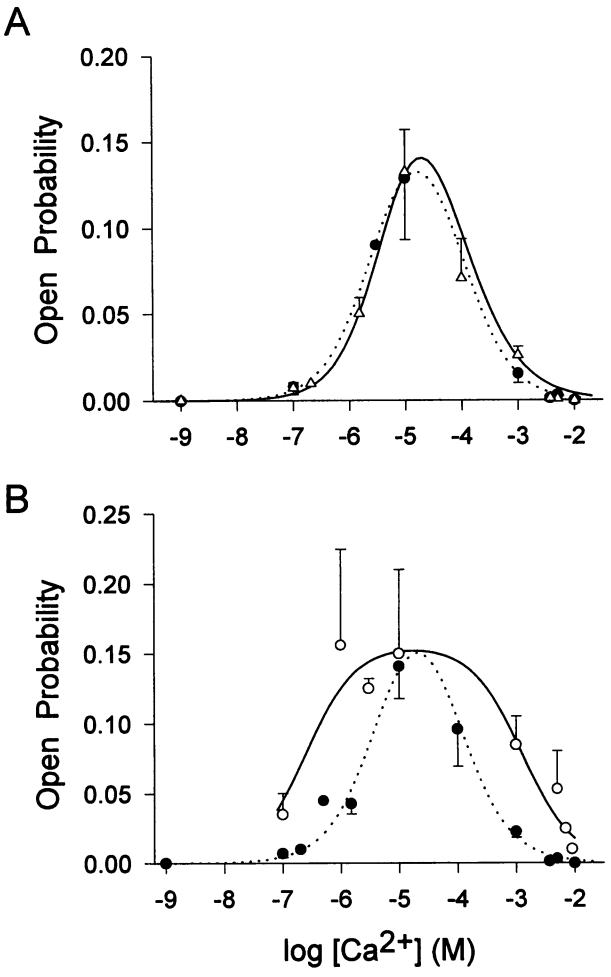

FIGURE 10 Dependence of open probability on *cis* $[Ca^{2+}]$ for RyR channels incorporated from native TC, control-incubated TC, and FKBP12-depleted TC. Usually, only one or two different *cis* Ca^{2+} concentrations could be tested per RyR channel within the lifetime of the bilayer. Open probabilities (P_o) were calculated (Methods) from ≥90 s of continuous channel records. *A*, Ca^{2+} sensitivity of the RyR was not significantly altered by the incubation procedure used to remove the FKBP12. Native RyR channel data (Δ, mean ± SE, n = 55) are compared with data of RyRs from control-incubated TC vesicles (●, mean ± SE, n = 26). The curves for native (*solid curve*) and for control-incubated (*dotted curve*) TC represent best least-squares fits to a modified Hill equation (Eq. 1 in Methods), which, with H_A and H_I set at 1.0, yielded values of K_A and K_I of 5.1 and 73 μM, respectively, for native channels and of 2.8 and 93 μM, respectively, for the control-incubated channels. *B*, Open probability of RyR channels from TC vesicles depleted of 75–100% of FKBP12 (○, mean ± SE, n = 50). Data from native and control-incubated channels in *A* were combined and plotted (●, mean ± SE, n = 81). The curves for FKBP12-depleted (*solid curve*) and control-plus-native (*dotted curve*) channels were fitted to the data in the same way as in *A*, with K_A and K_I of 4.9 and 89 μM, respectively, for the combined control channels and of 0.25 μM and 1.3 mM, respectively, for the FKBP12-depleted channels.

ined in detail. We found significant channel activity to three major subconductance levels at ~0.25, ~0.50, and ~0.75 of the maximum conductance, levels that were also reported for recombinant RyR in the absence of FKBP12 (Brillantes et al., 1994). In addition we found that the subconductance levels were maintained in the presence of micromolar ryanodine. The relative number of openings to or dwell time at each conductance level, or both, was differentially altered by *cis* $[Ca^{2+}]$ and 1 mM ATP. FKBP12-stripped channels, like normal channels, were modulated by *cis* $[Ca^{2+}]$: the Ca^{2+}-activation site displayed a higher-than-normal affinity for Ca^{2+}, whereas the Ca^{2+}-inhibition site had a lower affinity. Curiously, substate levels were not reported in multiple-channel activity of FKBP12-stripped RyRs recorded with 2 mM *cis* ATP (Mayrleitner et al., 1994).

Comparison of FKBP12-stripped RyRs and recombinant RyRs expressed in Sf9 cells

Although the relative subconductance levels in the FKBP12-stripped channels were the same as those in recombinant FKBP12-deficient RyRs purified from Sf9 cells (Brillantes et al., 1994), there were significant differences between the FKBP12-stripped and recombinant RyRs in other aspects of channel activity. First, recombinant RyRs (with or without FKBP12) were relatively insensitive to Ca^{2+} activation; they were not activated by 9 μM *cis* Ca^{2+} and were activated by 100 μM Ca^{2+} in only three of five experiments. In contrast, FKBP12-depleted RyR channels from skeletal muscle TC are strongly activated by 10 μM *cis* Ca^{2+} (this study and that of Mayrleitner et al., 1994). The reduced sensitivity of recombinant RyRs to activation by Ca^{2+} may be due to posttranslational processing, e.g., phosphorylation or glycosylation, which may differ between muscle and Sf9 cells and which is necessary for normal Ca^{2+} activation. The absence of a functional Ca^{2+}-activation site is not likely to be due to the absence of proteins associated with the RyR in TC vesicles because detergent-solubilized and -purified skeletal muscle RyRs are activated by micromolar *cis* Ca^{2+} (Smith et al., 1988; Lai et al., 1988).

The second striking difference between recombinant RyRs (Brillantes et al., 1994) and FKBP12-depleted channels is the proportion of activity to the maximum conductance. Channel openings to the maximum conductance were recorded in only 3 of 32 experiments by use of recombinant RyRs expressed without FKBP12 and were seen more frequently after addition of human recombinant FKBP12 but in only 40% of channels. In marked contrast, all RyR channels from FKBP12-depleted TC vesicles, including those that were ~100% stripped, opened to the maximum conductance. Posttranslational modification or interactions between the RyR and other bound proteins in addition to FKBP12, or both, may be required for "coordination" of the subconductance levels of the RyR and to allow channels to open fully. It would be necessary to purify FKBP12-depleted channels to assess these possibilities.

Discrete subconductance states, usually at ~0.25, ~0.5, and ~0.75 of the maximum conductance, have been ob-

TABLE 1 Effect of *cis* [Ca^{2+}] on the single-channel kinetics of control and FKBP12-depleted RyRs

	Control[#]			FKBP12 Depleted[§]			
[Ca^{2+}]	n	F_o (s^{-1})	T_o (ms)	T_c (ms)	n	F_o (s^{-1})	T_o (ms)	T_c (ms)
0.1 μM	8	3.8 ± 2.1	0.83 ± 0.09	983 ± 286	5	30 ± 13.3*	1.15 ± 0.19	128 ± 70*
10 μM	8	37.7 ± 16.0	14.05 ± 6.13	83 ± 27	5	48.1 ± 11.3	4.25 ± 1.26	53 ± 39
1 mM	22	15.5 ± 3.1	0.78 ± 0.05	160 ± 34	13	61.6 ± 11.9***	1.97 ± 0.48**	36 ± 14**

Values are shown as mean ± SE for n channels; F_o, number of events per second; T_o, mean open time; T_c, mean closed time. Values significantly different from control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ (Student's two-tailed t-test).
[#]Includes RyR channels from native and control-incubated TC.
[§]Includes RyR channels from TC depleted of 25–100% of FKBP12.

served in channel activity of detergent-solubilized and -purified skeletal muscle RyR (Lai et al., 1988; Ma et al., 1988; Smith et al., 1988; Hymel et al., 1988; Liu et al., 1989; Ma, 1993). The subconductance activity may have occurred because RyR channels lost bound FKBP12 during the purification (Wagenknecht et al., 1996; our unpublished observation). However, it is also likely that other TC proteins removed during purification are required for the coordination of channel opening to the full conductance.

Ligand binding influences the gating of FKBP12-depleted RyR channel activity to different conductance levels

The frequency of channel opening to the maximum level and to each subconductance state varied with experimental conditions. S1 was the most frequently observed level in continuous recordings (>15 s) from FKBP12-depleted channels that were either activated by micromolar *cis* Ca^{2+}

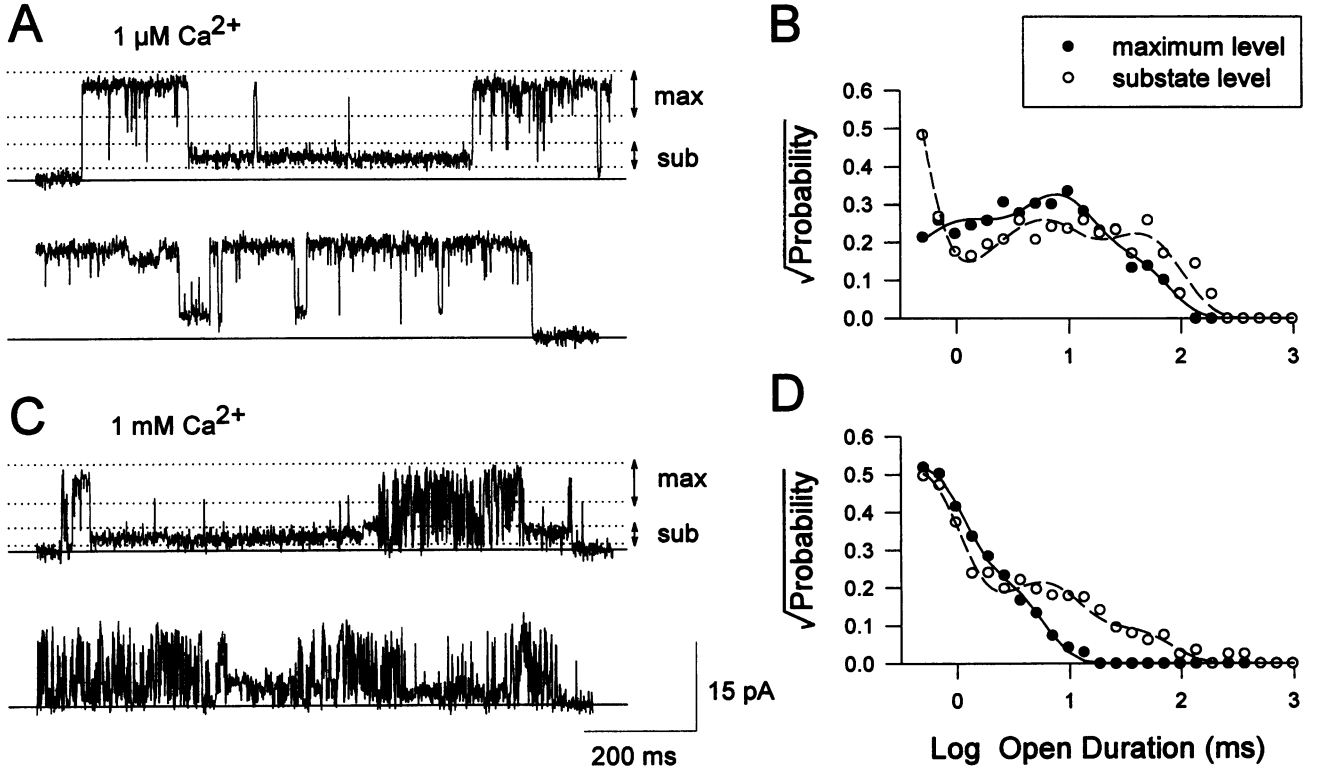

FIGURE 11 Maximum conductance and the S1 subconductance level in a single FKBP12-stripped RyR channel show different Ca^{2+} sensitivities. Representative activity of one channel at 1 μM *cis* Ca^{2+} (*A*) and 1 mM *cis* Ca^{2+} (*C*). The duration of events at the maximum and subconductance levels were compiled by detection of transitions into and out of the current windows (*A* and *C*, *dotted lines*) by use of the program EVPROC (see Methods). A window of 2–6 pA was assigned for the subconductance level in *A* and a window of 1–4 pA for the level in *C* (*two lowest dotted lines labeled sub*). Windows of 10.5–18 and 8–15 pA were assigned for the maximum open levels in *A* and *C*, respectively (*two upper dotted lines labeled max*) and include relatively infrequent openings to the S3 subconductance level. Open duration histograms for the maximum level (●) and the substate level (○) were plotted by the log-bin method (Sigworth and Sine, 1987). At 1 μM *cis* Ca^{2+} (*B*) the histograms were fitted to the sum of three exponentials (*continuous curves*). For the maximum level (*solid curve*) τ_1 = 0.7 ms, τ_2 = 5.9 ms, and τ_3 = 28.9 ms, and for the substate level (*dashed curve*) $\tau_1 < 0.5$ ms, τ_2 = 4.4 ms, and τ_3 = 40.4 ms. At 1 mM *cis* Ca^{2+} (*D*) the maximum level data were fitted with a double exponential (*solid curve*), $\tau_1 < 0.5$ ms, τ_2 = 2.2 ms, and the substate level data were fitted with the sum of three exponentials (*dashed curve*), $\tau_1 < 0.5$ ms, τ_2 = 4.7 ms, and τ_3 = 31 ms.

alone or inhibited by 1 mM *cis* Ca^{2+}. However, the maximum conductance was observed with increased frequency when 1 mM *cis* ATP was present in addition to the Ca^{2+}. Channel openings to S2 and S3 were more frequent in the presence of ATP, ryanodine, or both. A more even distribution of S1, S2, and S3 was observed when FKBP12-depleted RyRs were "fully activated" by ryanodine. Brillantes et al. (1994) found an even distribution of activity to the three submaximal conductance levels in recombinant FKBP12-deficient RyR channels activated by caffeine.

A differential sensitivity of conductance levels to ligands has been observed in a number of different channel types. High- and low-conductance states in a SR chloride channel have different sensitivities to Ca^{2+} activation (Kourie et al., 1996a) and to block by ATP (G. P. Ahern, unpublished observations) and inositol polyphosphates (J. I. Kourie et al., unpublished). Substate and maximum conductances in the large K^+ channel from *Chara* differ in their sensitivity to voltage (Tyerman et al., 1992), and acetylcholine receptors from rat myotubes show different sensitivities to block by curare (Takeda and Trautmann, 1984).

Effects of FKBP12 depletion on the cis Ca^{2+} sensitivity of RyRs

The FKBP12-depleted RyRs were more sensitive to Ca^{2+} activation than were normal channels in this study and in a report by Mayrleitner et al. (1994). In addition, we show here a decrease in the affinity of the Ca^{2+}-inhibition site that is consistent with a reduced Mg^{2+} sensitivity of Ca^{2+} release from FKBP12-stripped TC vesicles (Timerman et al., 1993, 1995) because both Ca^{2+} and Mg^{2+} are likely to bind to the same inhibitory site (see Meissner, 1994; Laver et al., 1997). The increase in P_o at 1 mM Ca^{2+} was found to be due largely the subconductance level S1, which showed long openings even in the presence of inhibiting $[Ca^{2+}]$.

The maximum RyR activation at 10 μM Ca^{2+} (measured by average P_o and by T_o) was not significantly altered by FKBP12 removal. This was in marked contrast to the effects of *cis* application of FK506 or rapamycin to native RyR channels, where P_o was higher than normal at all Ca^{2+} concentrations tested (Ahern et al., 1994). The difference may be due to a direct activation of the native RyR channel by the immunosuppressants in addition to the dissociation of the FKBP12 (Ahern et al., 1996). Alternatively, the FKBP12–rapamycin complex, which may remain bound to the RyR for a finite period before dissociating, could influence channel activity in a way that differs from simple FKBP12 removal.

Are submaximal conductance levels an inherent property of the skeletal RyR or are they induced by removal of FKBP12?

Subconductance levels were observed, albeit infrequently, in RyR channels that had not been stripped of FKBP12 (also reported by Liu et al., 1989). Infrequent and brief openings to subconductance levels were recorded from ~20% of native and control-incubated RyRs but were not usually seen as peaks in probability histograms of data from long continuous records. These observations could indicate that channel activity is occasionally uncoordinated, even when the RyR contains its full complement of four FKBP12 molecules. It is likely that the subconductance activity in the untreated channels is due to some transiently unoccupied FKBP12 binding sites. Timerman et al. (1995) estimated that 6% of FKBP12 binding sites are unoccupied in TC vesicles. We observed subconductance activity with low levels of FKBP12 depletion (<25%) and also shortly after addition of FK506 or rapamycin to native RyRs. Thus loss of only one FKBP12 molecule from the RyR tetramer may be sufficient to reduce the coordinated opening of the channel.

The structure of the RyR: Is there a physical basis for the conductance levels revealed by FKBP12 removal?

Three-dimensional reconstructions of cryoelectron micrographs of the frozen-hydrated RyR have been performed (Radermacher et al., 1992, 1994; Serysheva et al., 1995; Orlova et al., 1996). Radermacher et al. (1994) describe a central pore through the transmembrane assembly of the RyR, with a "plug" near its cytoplasmic mouth and four "canals" that radiate out through the cytoplasmic domains of the RyR. Orlova et al. (1996) have compared reconstructions of RyRs in the closed state and in a ryanodine-modified open state, observing a central pore through the transmembrane region in the open but not the closed state. They also detect pronounced differences in the structure of "clamp-like" domains at each of the four corners of the cytoplasmic assembly. Wagenknecht et al. (1996) demonstrated that FKBP12 is bound at the periphery of the RyR in four positions which appear to be directly adjacent to the "clamp-like" domains described by Orlova et al. (1996) and which are at least 10 nm away from the membrane spanning domain.

Wagenknecht and Radermacher (1995) suggest that the transmembrane region of the RyR appears to be connected to the cytoplasmic assembly by means of four symmetrically arranged domains that terminate near the "plug." Conformational changes induced by ligands binding to the extremities of the cytoplasmic assembly of the RyR, such as FKBP12 and calmodulin (Wagenknecht et al., 1994), could be transmitted through these four cytoplasmic domains to the transmembrane domains, as postulated by Wagenknecht and Radermacher (1995). As the RyR channel is voltage dependent (Ma, 1995; Laver et al., 1995), the gating regions are likely to be associated with the transmembrane domain. The four equally spaced subconductance levels in FKBP12-stripped RyR channels, each with similar ion selectivity, suggest that there may be an equivalent conducting pathway

associated with each RyR monomer. The binding of FKBP12, possibly its acting on the "plug" region through interconnecting protein domains, must in some way coordinate the four pathways in the native RyR such that they open and shut simultaneously.

Submaximal conductance levels in ryanodine-modified FKBP12-depleted RyRs

FKBP12-depleted and native RyRs were "locked" open by ryanodine, with the maximum conductance reduced by ~50%. The prominent substates in the FKBP12-depleted channels remained after ryanodine modification, with the amplitude of each level reduced by ~50%. Subconductance levels have also been reported in unstripped RyRs activated by nanomolar ryanodine (Buck et al., 1992; Ma, 1993; Ma and Zhao, 1994; Callaway et al., 1994).

Our observation, that the subconductance levels were reduced by the same amount as the maximum conductance, suggests that ryanodine binding reduces the maximum conductance by reducing equally the conductance of each of four parallel subconductance pathways. The FKBP12-stripped RyR can be modeled as an electrical circuit with four equivalent conductance pathways as resistors in parallel, each of which can be separately switched. Ryanodine binding can be assumed to insert either one resistor in series with the four parallel conductance pathways or four resistors, each in series with one conductance pathway. Simple circuit analysis shows that one resistor (induced by the binding of ryanodine) placed in series with four parallel conductance pathways could produce a 50% reduction in the maximum conductance, but the substate levels would not retain the fractional conductances seen in the absence of the resistance introduced by ryanodine. On the other hand, four added resistors, each in series with one of the four conducting pathways, would reduce the maximum and subconductance levels by the same proportion. The latter model is in agreement with the observed data but implies that ryanodine binds with equal affinity to a site in each of the four conducting pathways or that binding to one site induces equivalent conformational changes in each of the four subunits. The first implication is at odds with studies of ryanodine binding to native RyRs, which show one binding site with high (nanomolar) affinity (Buck et al., 1992; McGrew et al., 1989; Chu et al., 1990; Wang et al., 1993) that is likely to be located in the C terminus of the RyR in the region of the putative transmembrane helices (Witcher et al., 1994; Callaway et al., 1994). As yet there are no studies that investigate the binding of ryanodine to the FKBP12-stripped skeletal RyR channels to indicate whether there is any change in the number of high-affinity binding sites. Mack et al. (1994) measured binding of ryanodine in the presence of 50 μM FK506, which should be more than sufficient to remove all bound FKBP12 (Timerman et al., 1993). They found a 2–3-fold decrease in binding affinity and an increased capacity (B_{max}) for high-affinity [^{3}H]ryanodine binding but did not estimate the number of high-affinity sites per RyR. If there is no change in the number of high-affinity sites, then our results suggest that the binding of ryanodine to the stripped channel induces conformational changes in the RyR that could be transmitted to, and increase equally, the resistance of each of the four subconductance pathways.

Role of FKBP12 in RyR channel activity and E-C coupling

The present results reinforce the hypothesis that FKBP12 is crucial in coordinating the RyR tetramer so that it stabilizes the closed conformation (Timerman et al., 1993; Mayrleitner et al., 1994) and promotes transitions to the maximum channel conductance (Brillantes et al., 1994). As FKBP12 appears to be bound to the periphery of the cytoplasmic region, modulation of channel activity must be mediated by long-range allosteric effects (Wagenknecht et al., 1996). The conformation of RyR is likely to be altered by removal of FKBP12 (Slavik et al., 1996), although Wagenknecht et al. (1996) were unable to observe any gross changes.

Lamb and Stephenson (1996) postulated that FKBP12 could act as the link between the DHPR in the transverse-tubule membrane and RyRs during E-C coupling in skeletal membrane. They found that skinned muscle fibers, treated with rapamycin during repeated depolarizations, irreversibly lose their ability to contract in response to subsequent depolarization but not in response to the application of caffeine. The peripheral location of FKBP12 (Wagenknecht et al., 1996) is consistent with the hypothesis that FKPB12 acts as a link between the RyR and the DHPR. However, FKBP12 also binds to other receptors (e.g. IP_3 receptor, TGF-β receptor), which do not require the "mechanical" coupling between proteins in adjacent membranes that has been proposed for the skeletal muscle RyR and DHPR (Chandler et al., 1976; Block et al., 1988). The simplest hypothesis is that binding of four FKBP12 molecules stabilizes a conformation of the RyR that favors channel opening to the maximum conductance, as proposed by Brillantes et al. (1994), and that this conformation is required for "mechanical" activation of skeletal RyRs during E-C coupling. It has been proposed that the FKBP proteins, in general, may have evolved to play a functional role in signaling as components of multiprotein complexes (Kay, 1996); the interaction between RyR and FKBP12 would be an example of such a role.

To conclude, RyRs, depleted of FKBP12, have a higher P_o than normal channels at low *cis* [Ca^{2+}] (<1 μM) and at higher inactivating *cis* [Ca^{2+}] (>100 μM) but not at the optimal *cis* [Ca^{2+}] for channel activation (~10 μM). The FKBP12-depleted channels retain the maximum conductance of native RyRs but frequently open to additional subconductance levels. The three main subconductance levels remain after addition of micromolar *cis* ryanodine but are all reduced by ~50%. The results confirm that FKBP12

is essential in setting the normal gating properties of skeletal muscle RyR channel activity.

The authors wish to thank Dr. D. R. Laver and Prof. P. W. Gage for helpful discussion and comments on the manuscript, Dr. D. R. Laver for assistance with the EVPROC program, S. Pace and J. Stivala for help with TC vesicle isolation and antibody production, and M. Smith for assistance with programming.

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