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Rectification of Skeletal Muscle Ryanodine Receptor Mediated by FK506 Binding Protein

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ABSTRACT The cytosolic receptor for immunosuppressant drugs, FK506 binding protein (FKBP12), maintains a tight association with ryanodine receptors of sarcoplasmic reticulum (SR) membrane in skeletal muscle. The interaction between FKBP12 and ryanodine receptors resulted in distinct rectification of the Ca release channel. The endogenous FKBP-bound Ca release channel conducted current unidirectionally from SR lumen to myoplasm; in the opposite direction, the channel deactivated with fast kinetics. The binding of FKBP12 is likely to alter subunit interactions within the ryanodine receptor complex, as revealed by changes in conductance states of the channel. Both on- and off-rates of FKBP12 binding to the ryanodine receptor showed clear dependence on the membrane potential, suggesting that the binding sites of FKBP12 reside in or near the conduction pore of the Ca release channel. Rectification of the Ca release channel would prevent counter-current flow during the rapid release of Ca from SR membrane, and thus may serve as a negative feedback mechanism that participates in the process of muscle excitation-contraction coupling.

INTRODUCTION

In skeletal muscle, coupling of electrical excitation to mechanical contraction (E-C coupling) occurs in the junction between the transverse tubule (TT) and the sarcoplasmic reticulum (SR) membranes (Fleischer and Inui, 1989; Rios et al., 1991; McPherson and Campbell, 1993). Ryanodine receptors located in the terminal cisternae of the SR membrane play important roles in E-C coupling. The proteins span the junctional gap between TT and SR membranes (Wagenknecht et al., 1989; Takeshima et al., 1989; Zorzato et al., 1990), and they provide the conduction pathway for the Ca release channel (Smith et al., 1988; Lai et al., 1988; Ma et al., 1988). In addition to ryanodine receptors, other proteins, including triadin (Caswell et al., 1991; Knudson et al., 1993), calsequestrin (Ikemoto et al., 1989), and FK506 binding protein (FKBP12), also localize in the terminal cisternae of SR. FKBPs are ubiquitous receptors for immunosuppressant drugs (FK506 and rapamycin) (Schreiber, 1991), which bind stoichiometrically with the skeletal muscle ryanodine receptor (four FKBPs per tetramer of ryanodine receptors) (Jayaraman et al., 1992; Timerman et al., 1993). Biochemical studies indicated that dissociation of FKBP from ryanodine receptors enhanced the activity of the Ca release channel (Timerman et al., 1993), and co-expression of FKBP12 with ryanodine receptors in Sf9 cells led to stabilization of conductance states of the Ca release channel (Brillantes et al., 1994). However, the functional consequence of interaction between endogenous FKBP12 and the ryanodine receptor on the Ca release channel remains largely unknown.

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Chen et al. (1994) showed that when the recombinant FKBP12 was added to the purified ryanodine receptor from rabbit skeletal muscle, it induced asymmetric blockade of the Ca release channel. In the presence of exogenous FKBP12, the Ca release channel allowed only unidirectional flow of current from SR lumen to the myoplasmic space. With the isolated junctional SR membrane vesicles, we provide evidence that the endogenous FKBP12-bound Ca release channel also exhibits such distinct rectification.

MATERIALS AND METHODS

Isolation of junctional SR membrane vesicles

Junctional SR membranes were isolated from the rabbit skeletal muscle following the procedure of Meissner (1984). Briefly, fast twitch skeletal muscle was homogenized in 100 mM NaCl, 2 mM EDTA, 0.1 mM EGTA, and 5 mM TRIS-Maleate (pH 6.8). Microsome vesicles were obtained after sequential centrifugation at 2600 and 35,000 × *g*. The microsome vesicles were loaded onto a discontinuous sucrose gradient, and the junctional SR membranes were recovered from the 35–40% region of sucrose gradients. The vesicles were stored at −70°C at a concentration of 3–5 mg protein/ml until use.

Reconstitution of Ca release channel in lipid bilayer

Lipid bilayer membranes were formed across an aperture of ~200 μm diameter using the Muller-Rudin method with a mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1). The lipids were dissolved in decane at a concentration of 40 mg lipid/ml decane. The recording solution contained symmetrical 200 mM Cs-gluconate, 10 mM HEPES-Tris (pH 7.4). The free Ca concentrations in both *cis* and *trans* solutions were buffered with 1 mM EGTA. Incorporation of the Ca release channel in bilayer was achieved by addition of the SR vesicles (1–3 μl) to the *cis* solution, under a concentration gradient of 200 mM (*cis*)/50 mM (*trans*) Cs-gluconate.

Orientation of the channel in the lipid bilayer was always *cis*-myoplasmic, *trans*-luminal SR, as determined by sensitivity of the channel to *cis* Ca and ATP. Those channels with opposite orientation, which account for

<5% of the total experiments, were not used in the present study. The experiments were performed at room temperature (22–25°C).

The inward rectifying Ca release channel, which conducts current unidirectionally from SR lumen to myoplasm, was observed in four preparations of junctional SR vesicles. During the period from November 1993 to December 1994, we had a total of 190 successful experiments with the Ca release channel, of which at least 44 channels exhibited clear rectification.

Analyses of single-channel data

Single-channel currents were measured with an Axopatch 200A amplifier, in combination with a Digidata 1200 A/D-D/A conversion board (Axon Instruments, Foster City, CA). The records were filtered at a cutoff frequency of 2 kHz and digitized at a rate of 4–20 points/ms. The bilayers were held at a potential of 0 mV and pulsed to test voltages of various durations to measure the kinetics of deactivation and activation of the rectifying Ca release channel. Capacitance transients were subtracted from the test currents, using null sweeps as controls (Ma, 1995). The analyses of single-channel data were performed with the pClamp software. Bilayers with multiple channels were excluded from the analyses.

RESULTS

The isolated Ca release channel from rabbit skeletal muscle is a large conductance pore that is relatively nonselective for divalent over monovalent cations, with a permeability ratio of P_{Ca}/P_K ~6 (Smith et al., 1988). The channel had a linear conductance of 450 pS in a 200 mM symmetrical Cs-gluconate solution. A maximum open probability was measured when the myoplasmic solution contained 100 μM [Ca] and 2 mM ATP (Ma and Zhao, 1994). Cesium ions normally permeate through the Ca release channel equally in both the inward (SR lumen → myoplasm) and outward (myoplasm → SR lumen) directions, although the channel slightly favored the outward current, inasmuch as higher open probability was measured at positive voltages (+80 mV, Fig. 1 *A*). Such characteristics were observed in a majority of the experiments (146 of 190 experiments).

A distinct gating property was identified in a population of Ca release channels (~20% of the experiments). With alternate test pulses between negative and positive voltages, persistent channel activities could only be measured at −80 mV (Fig. 1 *B*). The ensemble average showed a clear tail current at +80 mV, which decayed with fast kinetics (τ_d = 35 ms) (Fig. 1 *B*, bottom trace). Thus the channel rectified in the inward direction of current flow. Other than rectification, the channel maintained all other characteristic properties of a Ca release channel. The opening of the channel

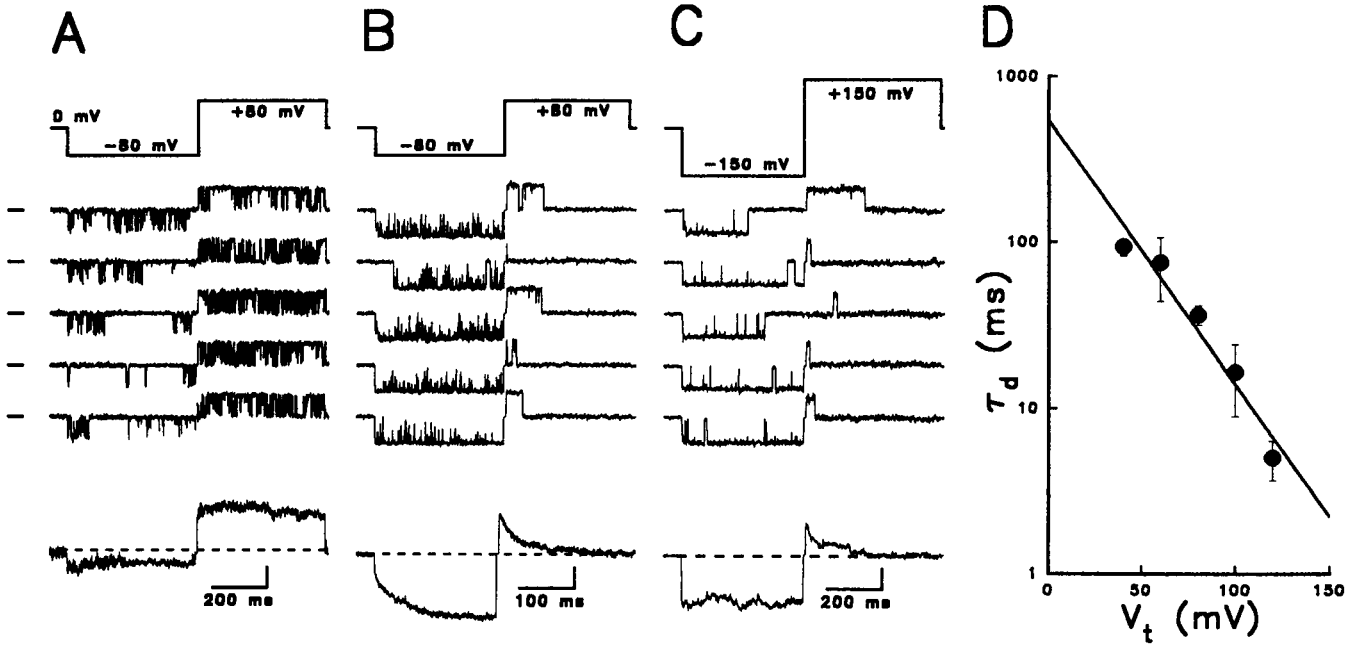

FIGURE 1 Voltage-dependent gating of a novel type of Ca release channel from rabbit skeletal muscle. Currents through single Ca release channels were measured in a symmetrical solution of 200 mM Cs-gluconate, 10 mM HEPES-Tris (pH 7.4). The free Ca concentration was 100 μM in the *cis*-myoplasmic solution and 6–10 μM in the *trans* solution (measured with a Ca-sensitive electrode). The *cis* solution contained, in addition, 2 mM ATP (Tris salt). (*A*) Consecutive episodes were taken from a normal Ca release channel at −80 mV and +80 mV (bilayer 94128). The potential was defined as *cis*-minus-*trans*. The channel exhibited higher open probability at positive voltage, as shown in the ensemble average. This behavior was observed in the majority of channels. (*B*) The novel type of Ca release channel, observed in ~20% of the experiments, allowed permeation of current only from SR lumen to myoplasm (at −80 mV) (bilayer 93D17). Ensemble average at +80 mV revealed a tail current, which decayed with a time constant of τ_d = 35.1 ± 6.3 ms. (*C*) The addition of 1 μM ryanodine reduced the channel conductance by ~50% and increased the open lifetime by >20-fold, without altering the rectification property of the Ca release channel. The tail current at +150 mV had a time constant of 53.0 ms. The vertical bars represent 30 pA for the single channel currents and 10 pA for the ensemble averages. (*D*) The deactivation time constants (τ_d) of current flow from myoplasm to SR lumen were plotted as a function of the test potential (V_t). Data were pooled from nine separate experiments (mean ± SE). The solid line was the best fit according to $\tau_d(V) = \tau_d(0)exp(-\delta zFV/RT)$, where $\tau_d(0)$ = 552 ± 12 ms and δz = 0.90 ± 0.04.

was sensitive to myoplasmic Ca, as reduction of Ca in the *cis* solution from 100 μM to 0.1 μM resulted in a significant decrease in channel open probability (Fig. 2 *C*), which suggested that the myoplasmic side of the channel faced the *cis* solution. On addition of 2 mM ATP to the *cis* solution, the channel activity increased more than 30-fold, from Po = 0.02 (−ATP) to Po = 0.70 (+ATP) at −80 mV (data not shown). Ryanodine at a concentration of 1 μM locked the channel into approximately half the full conductance state accompanied with slowing of channel gating kinetics. The binding of ryanodine, however, did not alter the rectification property of the Ca release channel (Fig. 1 *C*).

The time constant of deactivation (τ_d) for the outward current had a clear dependence on the membrane potential (V_t) (Fig. 1 *D*). The rate of deactivation at positive voltages decreased monotonically with the decrease in test potential. Essentially, at voltages <+30 mV, the channel opened constantly without apparent deactivation (during the 0.5-s duration of the test pulse). From the plot of τ_d versus V_t, the time constant of deactivation at 0 mV could be estimated at 552 ms. Also, the gating process sensed the membrane potential with an effective valence of δz = 0.90, i.e., the putative blocking particle binds to the channel pore at ~0.90/z deep into the SR lumen.

When 10 mM perchlorate (ClO4) was added to the myoplasmic solution, the rectifying Ca release channel became nonrectifying within 1–2 min (Fig. 3). Similar to that of a normal release channel (Fig. 1 *A*), persistent channel activity became evident at +80 mV (Fig. 2 *B*). ClO4 is a chaotropic anion that increases the activity and alters the conductance states of the skeletal muscle Ca release channel (Ma et al., 1993; Percival et al., 1994). ClO4 probably altered interactions between putative modulatory proteins and the rectifying Ca release channel. This putative interaction is unlikely to originate from the TT membrane, as subsequent addition of TT vesicles isolated from rabbit skeletal muscle (5–30 μg/ml) (Ma et al., 1991) had no effect on either the rectifying Ca release channel (n = 9) or the ClO4-modified Ca release channel (n = 6) (data not shown). Interestingly, the effect of ClO4 could be reversed by the addition of 0.3 μM FKBP12 to the myoplasmic solution (Fig. 3, after the second addition of FKBP12). In the presence of FKBP12, the channel rectified >90% of the time; only occasional late openings could be measured at the end of the +80 mV test pulse (Fig. 2 *D*). The concentration of FKBP12 used (0.3 μM) is sufficient to bind to the Ca release channel because the concentration of exogenous FKBP12 required to exchange native FKBP12 from SR has an EC_{50} of ~0.3 μM (Timerman et al., 1995).

The experiment shown in Fig. 4 further suggests that FKBP12 is involved in rectification of the Ca release channel. When FKBP12 was added to a normal (nonrectifying) Ca release channel, no significant changes in channel open probability were observed at either −80 or +80 mV (n = 20; Fig. 4 *B*). It is possible that the binding sites of FKBP that produced rectification of the native Ca release channel could not be easily accessed from the aqueous solution, as further addition of perchlorate to the myoplasmic solution produced similar inward rectification of the Ca release channel (n = 4; Fig. 4 *C*). The addition of perchlorate alone

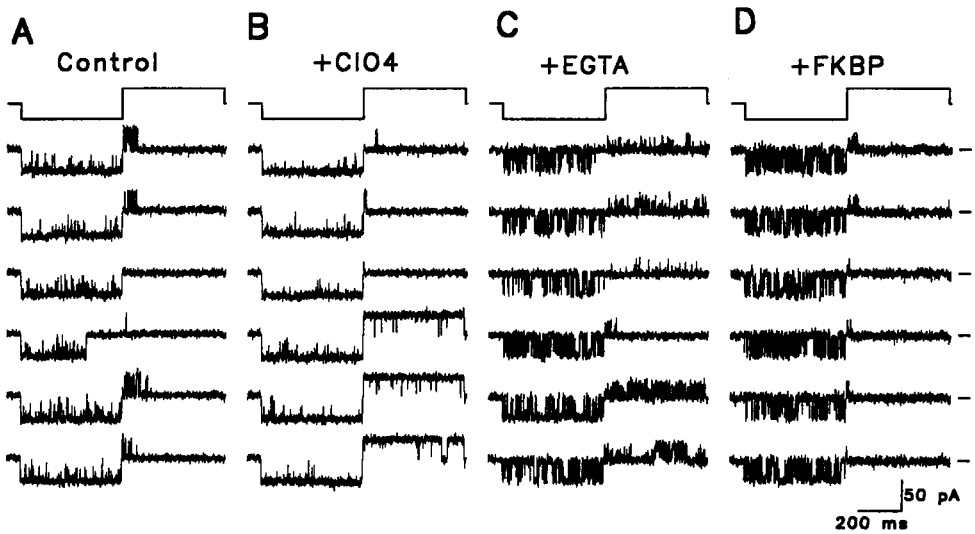

FIGURE 2 Effects of perchlorate and FKBP on the rectifying Ca release channel. Alternate test pulses from −80 to +80 mV were applied to the Ca release channel in bilayer. (*A*) Under control conditions with recording solution identical to Fig. 1, the channel rectified in the inward (SR lumen → myoplasm) direction. No persistent channel activity could be measured at +80 mV. (*B*) The addition of 10 mM perchlorate to the *cis* solution changed the rectification property of the channel, inasmuch as persistent channel activity became measurable at +80 mV. (*C*) The channel orientated in the bilayer in the *cis*-myoplasmic *trans*-luminal SR manner, as the addition of 2 mM EGTA to the *cis* solution resulted in a significant reduction of channel open probability at both −80 and +80 mV (see Fig. 3). (*D*) The subsequent addition of 0.3 μM recombinant FK506 binding protein (purchased from Sigma, St. Louis, MO) restored the rectification property of the Ca release channel. The traces were taken from one complete experiment (bilayer 94N03), which is representative of five other experiments. The effect of exogenous FKBP did not depend on the addition of EGTA, as it was observed in both 100 μM [Ca] (n = 4) and ~6 μM [Ca] (n = 2).

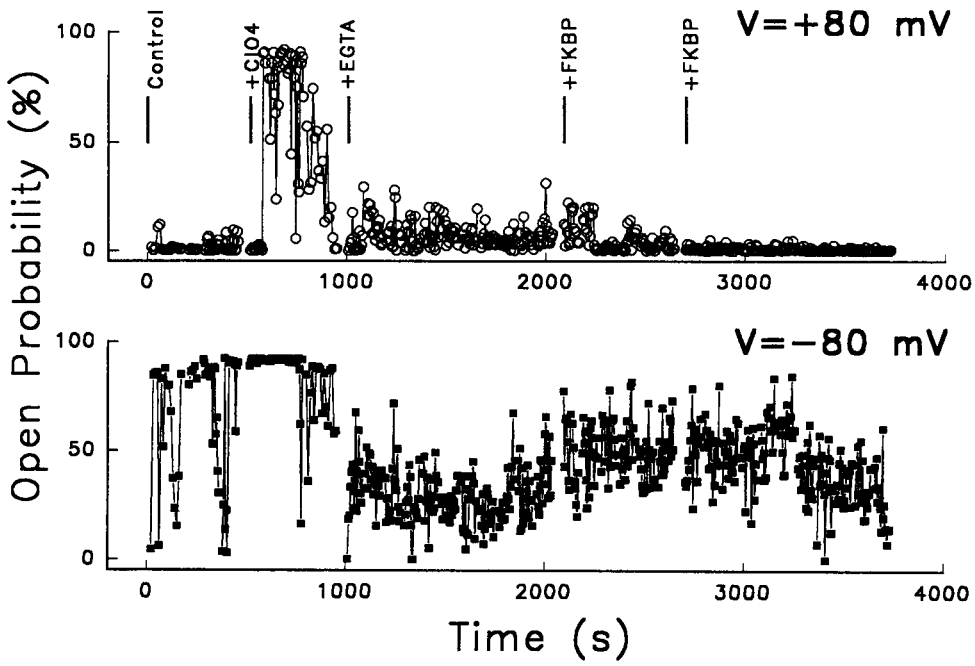

FIGURE 3 Diary plot of channel open probability. Open probabilities (*Po*) of the same bilayer experiment represented in Fig. 2 were plotted at +80 mV (*upper*) and −80 mV (*lower*). The data points represent the averaged *Po* over a duration of either 0.5 s or 2.5 s. The two additions of FKBP were both 0.15 μM.

to a normal Ca release channel did not produce rectification of the Ca release channel (n = 9). Thus FKBP is the molecule that is responsible for rectification of the Ca release channel.

Our result is consistent with the work of Chen et al. (1994), which showed that addition of exogenous FKBP12 to the purified ryanodine receptor induced asymmetrical blockade of the Ca release channel, leading to inward rectification of the single-channel current. To further examine the involvement of FKBP in the asymmetric gating of the Ca release channel, we tested FK506 on the rectifying Ca release channel (Fig. 5). Both the intrinsic rectifying Ca release channel (Fig. 5 *A*) and the FKBP-ClO4-mediated rectifying Ca release channel (Fig. 5 *B*) exhibited distinct sets of subconductance states on addition of 10 μM FK506 to the myoplasmic solution, similar to the results of Brillantes et al. (1994) and Ahern et al. (1994). However, the rectification property of the Ca release channel did not

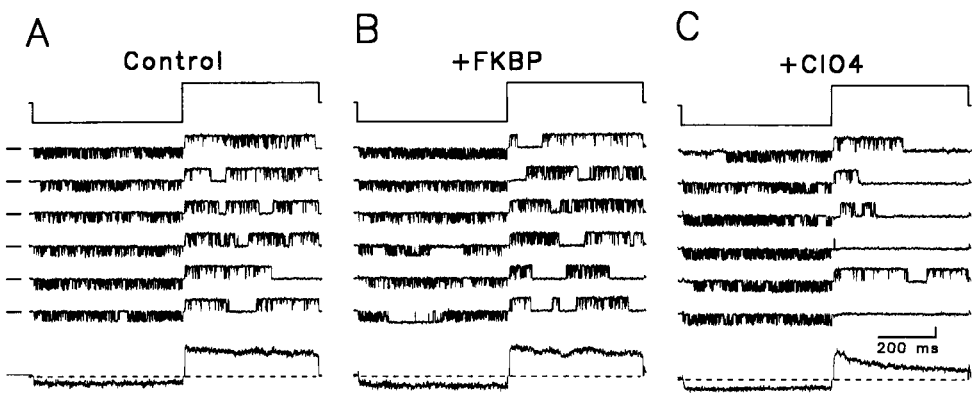

FIGURE 4 Effects of FKBP and perchlorate on the normal Ca release channel. The consecutive current episodes were taken from a complete experiment (bilayer 95626) with a normal (nonrectifying) Ca release channel under control conditions (same as in Fig. 1) (*A*), after the addition of 0.3 μM FKBP12 to the myoplasmic solution (*B*), and after the addition of 10 mM perchlorate to the myoplasmic solution (*C*). The corresponding ensemble averages are shown in the bottom (A = 80 episodes; B = 80 episodes; C = 240 episodes). The addition of exogenous FKBP12 to the normal Ca release channel did not result in significant changes in the channel open probability at either +80 or −80 mV (*B*). However, the channel exhibited clear inward rectification on further addition of ClO4 (*C*). The FKBP-mediated rectification of the normal Ca release channel in the presence of ClO4 was observed in four of seven experiments. Without perchlorate, the exogenous FKBP12 did not produce rectification of the Ca release channel (n = 20). The vertical bar represents 30 pA for single channel currents and 10 pA for ensemble averages.

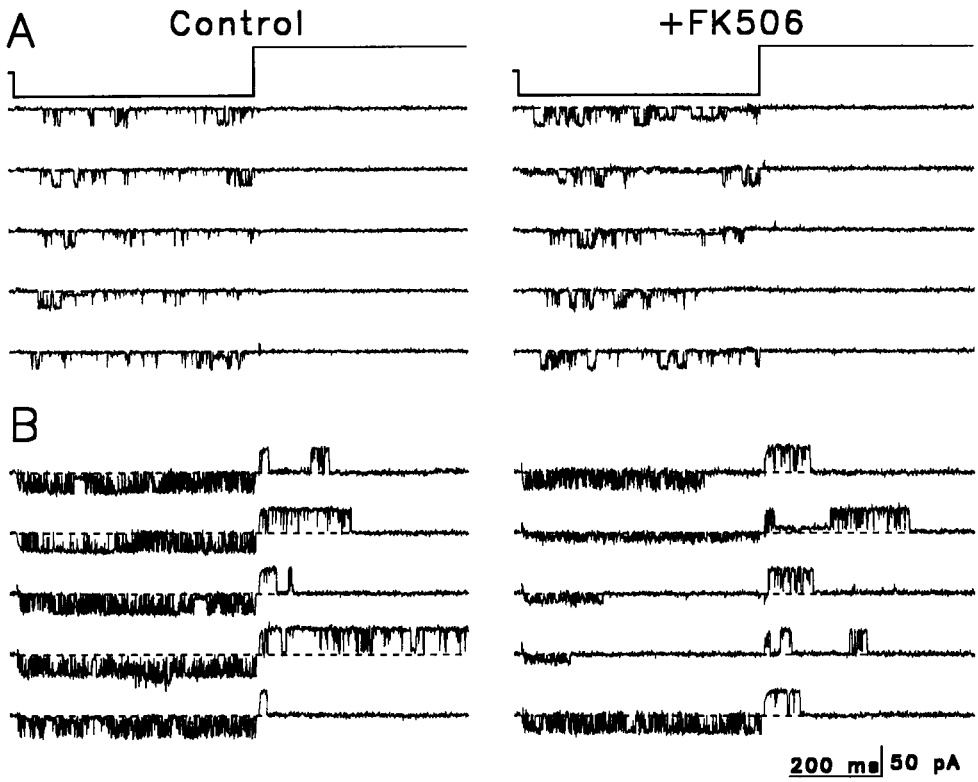

FIGURE 5 Effects of FK506 on the conductance states of the rectifying Ca release channel. Two separate sets of experiments were performed with FK506 on the intrinsically rectifying Ca release channel (*A*, bilayer 95622), and the ClO4-FKBP-mediated rectifying Ca release channel (*B*, bilayer 95628). The control condition for *A* was 2 mM ATP and 6 μM [Ca] in the myoplasmic solution. Notice the difference in channel open probability from those in Figs. 1 *B* and 2 *A*, where the concentration of Ca was 100 μM. The control condition for *B* (*left*) was 2 mM ATP and 100 μM [Ca], plus 10 mM ClO4 and 0.3 μM FKBP12. The additions of FK506 were 10 μM for *A* and for *B* (*right*). Notice the frequent appearance of subconductance states in the presence of FK506.

change significantly in the presence of FK506 (Fig. 5). Such a negative effect of FK506 on the rectification property of the Ca release channel was also observed by Chen et al. (1994), the implication of which will be presented in the Discussion.

Close examination revealed that opening of the rectifying Ca release channel contained intermediate transition steps in response to changes in membrane potential. Traces shown in Fig. 6 were obtained from an inward rectifying Ca release channel in the presence of 1 μM ryanodine at -100 mV (*A*) and -60 mV (*B*). It is clear that the putatively FKBP-bound Ca release channel exhibits multiple transitions before conversion to the full ryanodine-modified conductance state. These transitions were highly voltage-dependent, with a δz value of 0.80 (Fig. 6 *C*), similar to the deactivation process (Fig. 1 *D*). A possible explanation for the unstable transitions of the Ca release channel is the dissociation of FKBP from the ryanodine receptor at negative voltages, inasmuch as such a slow conversion process seemed to be a particular property of the rectifying Ca release channel. In 32 of 44 experiments with the rectifying Ca release channel, clear unstable transitions were observed ($n = 22$, without ryanodine, as shown in Fig. 1 *B*; $n = 10$, in the presence of 1 μM ryanodine, as shown in Fig. 6). In contrast, such unstable transitions were rarely seen with the normal (nonrectifying) Ca release channels ($n = 2$ of 146 experiments).

DISCUSSION

We have shown that the skeletal muscle Ca release channel, when bound to the FKBP12, exhibits clear rectification; the channel allows unidirectional flow of currents from the SR lumen to the myoplasmic space. The interaction of the ryanodine receptor with the endogenous FKBP12 was sensitive to the treatment of a chaotropic anion, perchlorate. The normal (nonrectifying) Ca release channel, when treated with perchlorate, exhibited similar inward rectification in the presence of exogenous FKBP12. With the voltage-clamped pulse protocols, we showed that the on- and off-rates of FKBP12 binding to the ryanodine receptor was voltage dependent. Thus the binding site of FKBP is likely to reside in or near the conduction pore of the Ca release channel.

FK506, as a potent ligand for FKBP12, would affect the binding of FKBP12 to the ryanodine receptor (Timerman et al., 1993). Consequently, this may be responsible for the distinct subconductance states observed in a single Ca re-

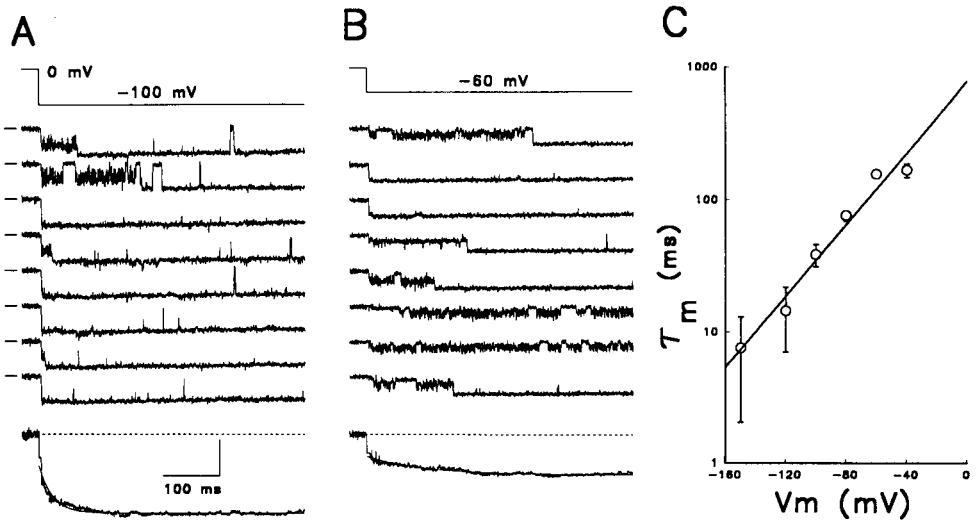

FIGURE 6 Intermediate close-open transitions of the rectifying Ca release channel at negative voltages. An inward rectifying Ca release channel was incorporated into the bilayer membrane, in the presence of 1 μM ryanodine. The ryanodine-activated channel had longer open lifetimes, which allowed better resolution of the intermediate transition steps. Consecutive episodes were represented at −100 mV (*A*) and −60 mV (*B*), from a holding potential of 0 mV (bilayer 93D03). Notice the initial unstable open transitions. The time constants of transition (τ_m) could be estimated from the ensemble averages: τ_m = 23.15 ± 5.02 ms and 96.87 ± 2.05 ms at −100 mV and −60 mV, respectively. The voltage dependence of τ_m is shown in *C*. The solid line represents the best fit according to $\tau_m(V) = \tau_m(0)exp(\delta zFV/RT)$, where $\tau_m(0)$ = 774 ± 22 ms and δz = 0.80 ± 0.07. The vertical bar represents 30 pA for single channel currents and 10 pA for ensemble averages.

lease channel in the presence of FK506 (Ahern et al., 1994). However, FK506 was without effect on the rectification property of the Ca release channel (Fig. 5). The separate effects of FK506 on the conductance states and rectification of the Ca release channel suggest that different mechanisms (binding of FKBP to the ryanodine receptor and blocking of the Ca release channel) are likely to be involved in the interaction of FKBP with the Ca release channel.

One possible interpretation is that FKBP12 acts as a tethered ligand to the ryanodine receptor, which exerts its effect on the Ca release channel through the movement of charged portions of the molecule in and out of the membrane field, as first suggested by Chen et al. (1994). Examination of the primary sequence of human FKBP12 revealed that there is a region of positively charged amino acids (a.a. 33 to 50) (Standaert et al., 1990). It will be interesting to know which specific domain of FKBP12 is responsible for the binding and interaction with the ryanodine receptor, and which region of FKBP12 is responsible for the voltage-dependent block of the ion conduction pathway of the Ca release channel. The existence of quasi-stable states for the rectifying Ca release channel suggests that binding of FKBP12 is likely to alter subunit interactions within the ryanodine receptor complex (Snyder and Sabatini, 1995). Other examples in which cytosolic ligands modulate the function of membrane proteins have been reported with the inward rectifier K channel, where polyamines such as spermine and spermidine served as intrinsic gating particles that produced a voltage-dependent block of the outward K current (Ficker et al., 1994; Lopatin et al., 1994).

The block of the perchlorate-treated nonrectifying Ca release channel by exogenous FKBP12 seemed to occur on a slower time course than the deactivation time course of the endogenous FKBP-bound Ca release channel (compare Fig. 4 *C* with Fig. 1 *B*). An alternative explanation for the slow time course of block is that the treatment of perchlorate allows the added FKBP12 to act as a small diffusible blocker of the Ca release channel pore without necessitating binding of the FKBP12 protein to the ryanodine receptor. Additional experiments are necessary to distinguish the mechanisms of "tethered binding" versus "diffusible blocking" of the Ca release channel by exogenous FKBP12. For example, by washing out the added FKBP12 after it blocks the channel, the blocking effect of FKBP12 should persist if FKBP12 occupies its specific binding sites on the ryanodine receptor. However, we were not successful at perfusing the bilayer solutions because of technical difficulties.

The arrival of action potential to the TT membrane induced rapid release of Ca from the SR, which in turn caused transient changes in electric potential across the SR membrane from ~0 mV (Somlyo et al., 1977) to near the equilibrium potential for Ca. This potential change is largely balanced by the counter current flow of K ions into the SR membrane through the SR potassium channel (Coronado et al., 1980) and the Ca release channel. Inward rectification of the Ca release channel by FKBP may serve as a negative feedback mechanism that slows the recovery of SR membrane potential, thus decreasing the rate of additional Ca release from SR. An understanding of the mechanism and the site of FKBP interaction with the Ca release channel should provide further insights into the structure and function of ryanodine receptors and their role in the regulation of muscle contraction.

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