

FKBP12.6 Activates RyR1: Investigating the Amino Acid Residues Critical for Channel Modulation

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ABSTRACT We have previously shown that FKBP12 associates with RyR2 in cardiac muscle and that it modulates RyR2 function differently to FKBP12.6. We now investigate how these proteins affect the single-channel behavior of RyR1 derived from rabbit skeletal muscle. Our results show that FKBP12.6 activates and FKBP12 inhibits RyR1. It is likely that both proteins compete for the same binding sites on RyR1 because channels that are preactivated by FKBP12.6 cannot be subsequently inhibited by FKBP12. We produced a mutant FKBP12 molecule (FKBP12_{E31Q/D32N/W59F}) where the residues Glu³¹, Asp³², and Trp⁵⁹ were converted to the corresponding residues in FKBP12.6. With respect to the functional regulation of RyR1 and RyR2, the FKBP12_{E31Q/D32N/W59F} mutant lost all ability to behave like FKBP12 and instead behaved like FKBP12.6. FKBP12_{E31Q/D32N/W59F} activated RyR1 but was not capable of activating RyR2. In conclusion, FKBP12.6 activates RyR1, whereas FKBP12 activates RyR2 and this selective activator phenotype is determined within the amino acid residues Glu³¹, Asp³², and Trp⁵⁹ in FKBP12 and Gln³¹, Asn³², and Phe⁵⁹ in FKBP12.6. The opposing but different effects of FKBP12 and FKBP12.6 on RyR1 and RyR2 channel gating provide scope for diversity of regulation in different tissues.

INTRODUCTION

FK506-binding proteins (FKBPs) bind tightly to ryanodine receptor (RyR) channels in cardiac and skeletal muscle, and evidence suggests that they play an important regulatory role that is impaired in disease, for example, in heart failure (1–4) and certain skeletal muscle disorders (5). It is reported that FKBPs provide a stabilizing effect on RyR channel function by lowering open probability (P_o) and preventing subconductance state gating and that, at the cellular level, this leads to fewer leaky RyR channels and fewer aberrant Ca^{2+} -release events (6–8). It is generally thought that FKBP12 stabilizes RyR1 and FKBP12.6 stabilizes RyR2 (1,9–11). This is a controversial area, however, because not all investigators agree that low P_o or substate gating in RyR channels is dependent on the binding of FKBPs (12–15). Moreover, we have recently shown that FKBP12 binds with high affinity to RyR2, causing channel activation and stimulating Ca^{2+} -induced Ca^{2+} -release in isolated cardiac myocytes (15). We demonstrated that FKBP12.6 cannot itself lower RyR2 P_o , but because it is a partial agonist of very low efficacy, it can act as an antagonist of FKBP12 and indirectly reduce RyR2 P_o and sarcoplasmic reticulum

(SR) Ca^{2+} -release in cardiac cells. Thus, the gating of RyR2 in cardiac muscle is under the dual modulation of FKBP12 and FKBP12.6, rather than the exclusive influence of FKBP12.6.

Other experiments also suggest that FKBP12 and FKBP12.6 do not selectively regulate RyR1 and RyR2, respectively. In an FKBP12 knockout mouse model, skeletal muscle function appeared normal, whereas cardiac hypertrophy and disturbances in cardiac excitation-contraction coupling were very severe (16). It has also been shown that FKBP12.6 can affect skeletal muscle function (17–19) indicating that, similar to RyR2 in cardiac muscle, RyR1 may also be subjected to dual regulation by FKBP12 and FKBP12.6.

We have therefore investigated how FKBP12 and FKBP12.6 affect the single-channel behavior of RyR1 and, to understand the mechanisms underlying their functional effects, we have made direct comparisons of their effects on RyR2 gating. FKBP12 and FKBP12.6 share the same number of amino acids (108) and with 82% sequence identity have similar molecular masses (11.8 kDa, FKBP12.6; 11.9 kDa, FKBP12 (15)). X-ray crystallography highlights the close structural similarity of the two proteins, providing few clues about the residues that may be important for binding to RyR1 and RyR2 or for transducing distinct functional effects. Previous work has indicated that the residues Gln³, Arg¹⁸, and Met⁴⁹ are necessary for FKBP12 binding to RyR1 (20). Likewise, mutation of Asp³⁷ to Val or Ser is reported to enhance the affinity of FKBP12.6 for RyR2 (21,22). Although affinity is important, the particular amino acid residues important for efficacy

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(the ability of FKBP12/12.6 to act as agonist or antagonist of RyR1/RyR2) have not been investigated previously. By locating the steric and electrostatic differences between FKBP12 and FKBP12.6 we identified three amino acid residues that could be relevant in this respect. We generated a mutant FKBP12 molecule (FKBP12_{E31Q/D32N/W59F}), in which these three residues were mutated to the corresponding residues found in FKBP12.6 and investigated if the mutant possessed increased or decreased ability to act as an activator (agonist) of RyR1 and/or RyR2.

We find that FKBP12 is an inhibitor of RyR1 but an activator of RyR2. Conversely, FKBP12.6 is an activator of RyR1 but has barely detectable ability to activate RyR2. The triple mutant, FKBP12_{E31Q/D32N/W59F}, lost all ability to activate RyR2 and inhibit RyR1 but instead, caused significant activation of RyR1. Thus, the ability for conferring selectivity of RyR agonist behavior is contained within a few key amino acids.

MATERIALS AND METHODS

Isolation of membrane fractions and bilayer techniques

Heavy sarcoplasmic reticulum (HSR) vesicles were obtained from sheep hearts (collected from an abattoir) or rabbit skeletal muscle, as described previously (23). SR vesicles were fused with planar phosphatidylethanolamine lipid bilayers as described (23). Incorporation of RyR always occurred in the same fixed orientation with the *cis* side corresponding to the cytosolic channel side and the *trans* side to the luminal face. The *trans*-chamber was held at ground and the *cis*-chamber clamped at potentials relative to ground. After fusion, the *cis*-chamber was perfused with 250 mM HEPES, 80 mM Tris, 10 μ M free Ca^{2+} , pH 7.2. The *trans*-chamber was perfused with 250 mM glutamic acid and 10 mM HEPES, pH to 7.2 with $\text{Ca}(\text{OH})_2$ (free $[\text{Ca}^{2+}]$, ~50 mM). Experiments were performed at $22 \pm 2^\circ\text{C}$. Free $[\text{Ca}^{2+}]$ and pH were maintained constant during experiments and were determined using a Ca^{2+} electrode (Orion 93–20, Thermo Fisher Scientific Inc., Waltham, MA) and Ross-type pH electrode (Orion 81–55, Thermo Fisher Scientific Inc., Waltham, MA) as previously described (23). FKBP12 and FKBP12.6 were added to the *cis*-chamber. Both proteins were stored in a buffer containing 10 mM HEPES, 50 mM NaCl, 0.5 mM DTT and volumes added to the *cis*-chamber were <2% of the total volume. Control experiments demonstrated that the buffer alone caused no effects on RyR function. Rapamycin treatment of the skeletal HSR membrane fraction was obtained by incubating vesicles with 20 μ M rapamycin for 15 min at 36°C . Following incubation, the membrane fraction was sedimented at $180,000 \times g$ for 15 min at room temperature.

Data acquisition and analysis

Single-channel currents were monitored under voltage-clamp conditions using a BC-525C amplifier (Warner Instruments, Hamden, CT). Channel recordings were low-pass filtered at 10 kHz with a 4-pole Bessel filter, digitized at 100 kHz using an ITC-18 data acquisition interface (HEKA Elektronik, Lambrecht/Pfalz, Germany) and recorded on a computer hard drive using WinEDR 3.05 software (John Dempster, University of Strathclyde, UK). The recordings were subsequently filtered at 800 Hz (–3 dB) using a low-pass digital filter implemented in WinEDR 3.05. Channel events were detected by the 50% threshold method (24) using TAC 4.2.0 software (Bruxton, Seattle, WA). Po and lifetime distributions were calculated from 3 min of continuous recording using TACfit 4.2.0 software (Bruxton). Po

diary plots were obtained using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA).

Preparation of wild-type and mutant FKBP12

Human FKBP12 and rabbit FKBP12.6 were cloned, expressed, and purified as previously described (15). The preparation of FKBP12_{E31Q/D32N/W59F} triple mutant is described in the Supporting Material. FKBP12 was also purchased from Sigma-Aldrich (Dorset, UK).

Statistics

Data are expressed as mean \pm SE where $n \geq 4$. For $n = 3$, SD is given. Where appropriate, Student's *t*-test was used to assess the difference between treatments. Where multiple treatments were compared, analysis of variance followed by a modified *t*-test was used to assess the difference between treatments. A *p* value of <0.05 was taken as significant.

Materials

All chemicals were obtained from VWR (Poole, UK) or Sigma-Aldrich. All solutions were prepared in MilliQ deionized water (Millipore, Harrow, UK) and those for use in bilayer experiments were filtered through a Millipore membrane filter with 0.45 μ m pore diameter.

RESULTS

We have previously demonstrated that both FKBP12 and FKBP12.6 behave as very high affinity, partial agonists of RyR2 (15). FKBP12 activates RyR2 at low picomolar concentrations. In contrast, the stimulatory effects of FKBP12.6 on RyR2 are usually imperceptible because although FKBP12.6 can bind to RyR2, it has extremely low efficacy. The Supporting Material (Fig. S1) shows typical examples of how FKBP12 and FKBP12.6 affect RyR2 gating. To make direct comparisons, identical recording conditions were used to investigate the effects of FKBP12 and FKBP12.6 on rabbit skeletal RyR1 gating. Unexpectedly, FKBP12.6 caused an increase in RyR1 Po. This effect was irreversible on the timescale of a single-channel experiment as washout of protein from the cytosolic chamber did not lower Po (Fig. 1 A). The irreversibility of the FKBP12.6 effect suggests a high affinity interaction between FKBP12.6 and RyR1 and this is confirmed by the fact that concentrations as low as 10 pM FKBP12.6 can significantly increase Po. Fig. 1 B shows the mean data for different concentrations of FKBP12.6. The mean open and closed times derived from experiments where only single channels were present in the bilayer were 1.68 ± 0.35 ms and 91.2 ± 34.9 ms, respectively, before and 2.26 ± 0.27 ms and 58.4 ± 37.3 ms (SD; $n = 3$), respectively, after addition of 200 nM FKBP12.6 indicating that FKBP12.6 primarily increases channel opening frequency with little effect on open lifetime duration. Lifetime analysis (see Fig. S2) confirms this mechanism of action; the open lifetime distribution is not altered even at high concentrations of FKBP12.6.

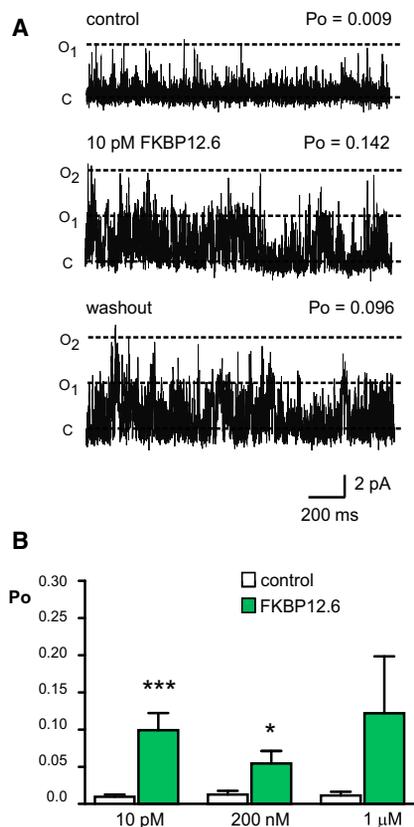


FIGURE 1 FKBP12.6 activates rabbit skeletal RyR1. (A) A typical single-channel experiment showing marked activation of RyR1 by 10 pM FKBP12.6. The bottom trace shows washout of FKBP12.6 from the cytosolic chamber. P_o values are indicated. Dashed lines indicate open (O_1 , O_2) and closed (C) channel levels, respectively. (B) Mean P_o data obtained before and after addition of 10 pM, 200 nM, and 1 μM FKBP12.6 (SE; $n = 5$ –11; *** $p < 0.001$; * $p < 0.05$). Each concentration represents a set of independent experiments. To see this figure in color, go online.

In contrast, FKBP12 always reduced the P_o of RyR1. A typical experiment is shown in Fig. 2 A with mean data shown in Fig. 2 B. Washout of cytosolic solutions to remove unbound FKBP12 did not reverse the inhibition as shown in the bottom trace. In four experiments where FKBP was washed out and P_o was recorded for a further 3 min, P_o was 0.007 ± 0.005 before and 0.002 ± 0.001 (SE; $n = 4$) after washout. The diary plot of channel activity during a typical experiment is shown in Fig. 2 C. This illustrates the modal gating that is characteristic of RyR channels (25,26) and the irreversibility of the effect of FKBP12. Detailed lifetime analysis was not possible because of the low number of events that occurred after addition of FKBP12. However, mean open and closed times were 2.41 ± 0.16 ms and 22.9 ± 3.94 ms (SE; $n = 4$), respectively, before and 2.04 ± 0.04 ms and 332 ± 142 ms (SE; $n = 4$), respectively, after the addition of 500 nM FKBP12 showing that the main effect of FKBP12 is to reduce channel opening frequency.

It is generally assumed that FKBP12.6 is not important for RyR1 function in skeletal muscle because FKBP12.6 is pre-

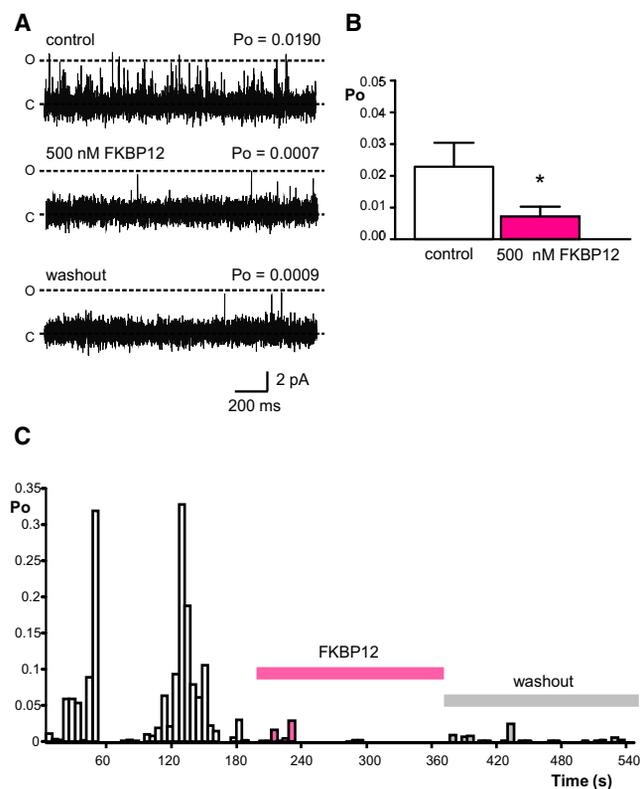


FIGURE 2 Inhibitory effects of FKBP12 on rabbit skeletal RyR1. (A) A representative experiment showing that RyR1 P_o is reduced by 500 nM FKBP12. The bottom trace shows that perfusion of the *cis* chamber back to control solutions did not reverse the reduction in P_o . The dashed lines indicate open (O) and closed (C) channel levels, respectively. P_o values are indicated above each trace. (B) Mean data showing that 500 nM FKBP12 inhibits RyR1 activity (SE; $n = 8$; * $p < 0.05$). (C) Diary plot showing RyR1 P_o in the presence of 10 μM cytosolic Ca^{2+} (control), after addition of 500 nM FKBP12 and after *cis* chamber perfusion back to control solutions. Single-channel traces were subdivided into 10 s sections and P_o was measured for each section and plotted against time. Note that even during control periods, RyR1 channels display marked variability of gating over time with random switching between low and high P_o modes. The bars indicate the times of incubation with FKBP12 and washout of FKBP12. To see this figure in color, go online.

sent at very low levels, whereas FKBP12 is present at micromolar levels (1–3 μM) (12,27,28). The data shown in Fig. 1, however, indicates that activation of RyR1 results from a high affinity interaction with FKBP12.6 and so an important question to answer is whether FKBP12 and FKBP12.6 could compete for the same binding sites. If so, then preaddition of FKBP12.6 should prevent FKBP12 from causing the immediate inhibition of RyR1 that is shown in Fig. 2 C. Fig. 3 A and B confirm that this is the case. Preaddition of FKBP12.6 (200 nM) prevents FKBP12 (500 nM) from causing channel inhibition (on the timescale of a single-channel experiment). There are two further issues to consider. 1), If the affinities of FKBP12/FKBP12.6 for RyR1 are as high as Figs. 1 and 2 suggest and the dissociation of FKBP from RyR1 is very slow, then even picomolar quantities of either isoform should prevent the binding of the

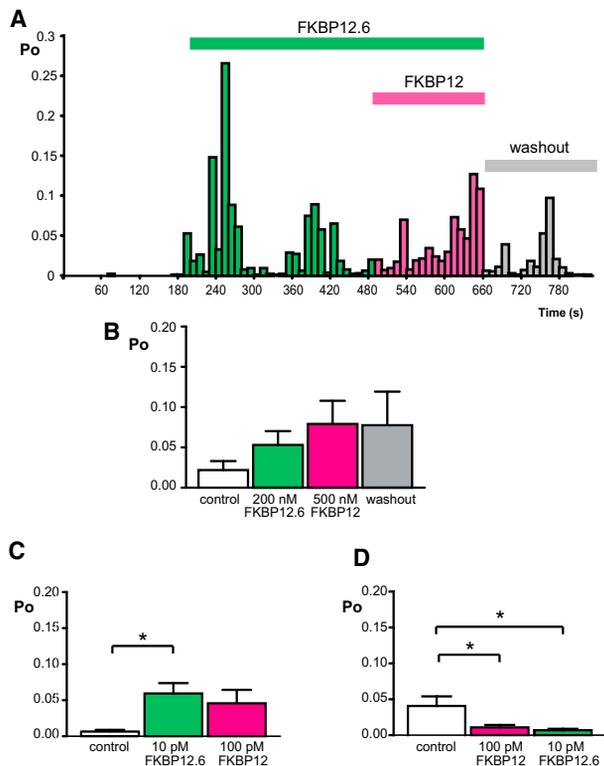


FIGURE 3 Preaddition of FKBP12.6 prevents the inhibition of rabbit skeletal RyR1 by FKBP12. (A) A representative diary plot of RyR1 P_o changes following preincubation with 200 nM FKBP12.6 and subsequent addition of 500 nM FKBP12 (incubation periods are shown by the bars). Control solutions contained 10 μ M cytosolic Ca^{2+} as the sole channel activator. Subsequent addition of 500 nM FKBP12 did not override the activating effects of FKBP12.6. After washout of the *cis* chamber, P_o was not completely reversed to control values. (B) Bar chart showing the mean P_o data for control, preaddition of 200 nM FKBP12.6, subsequent addition of 500 nM FKBP12, and washout to control solutions (SE; $n = 4$). Pretreatment with picomolar levels of FKBP12.6 (C) or FKBP12 (D) at the 10:1 ratio of FKBP12:FKBP12.6 that is expected in situ, was able to block the subsequent addition of the other FKBP isoform (SE; $n = 10$ –11; $*p < 0.05$). To see this figure in color, go online.

other isoform. 2), Physiological levels of FKBP12 are likely to be at least 5–10 times the levels of FKBP12.6, therefore, what happens in the competition studies if FKBP12 is present at 10 times the levels of FKBP12.6? To address these questions, we have performed experiments at 10 pM FKBP12.6 and 100 pM FKBP12 (pretreating first with 10 pM FKBP12.6 (Fig. 3 C) or 100 pM FKBP12 (Fig. 3 D). The experiments demonstrate pharmacological consistency; the high affinity binding and slow dissociation rate of either isoform of FKBP blocks the binding of the subsequently added isoform of FKBP.

To identify the amino acid residues in FKBP12 that are important for the selective ability of FKBP12 to reduce the P_o of RyR1 and increase the P_o of RyR2, we compared the specific amino acids of FKBP12 and FKBP12.6 that differ. Fig. 4 compares the primary sequences of FKBP12 and FKBP12.6 and their three-dimensional structures

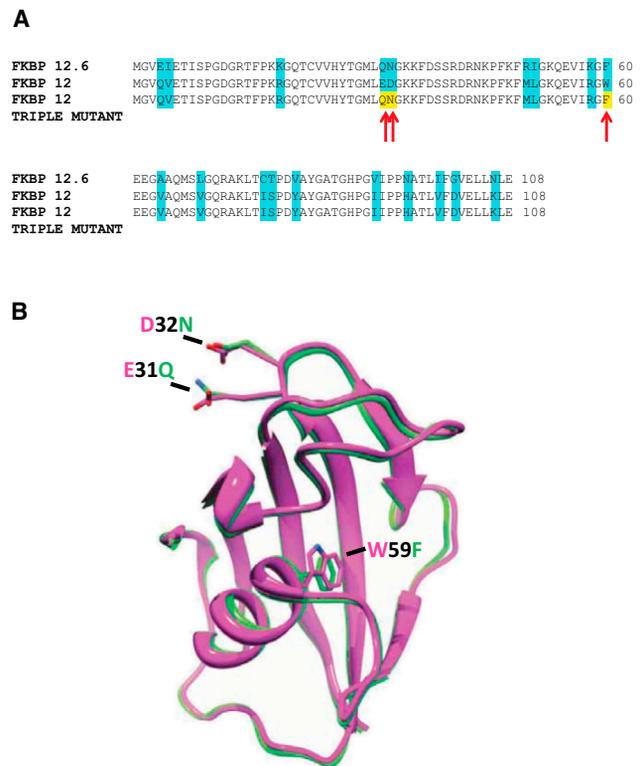


FIGURE 4 Comparison of FKBP12 and FKBP12.6 proteins to highlight the amino acid substitutions of the FKBP12_{E31Q/D32N/W59F} mutant. (A) Amino acid sequence alignments of human FKBP12, human FKBP12.6, and FKBP12_{E31Q/D32N/W59F}. The residues of FKBP12 and FKBP12.6 that differ are highlighted in blue. The red arrows indicate the three amino acid residues that were substituted into the FKBP12_{E31Q/D32N/W59F} mutant. (B) Overlaid ribbon diagrams of human FKBP12 and human FKBP12.6 based on their crystal structures (RCSB PDB accession codes 2DG3 and 1C9H, respectively) showing the three residues highlighted as most likely relevant amino acid substitutions between the two proteins. The dashes indicate these residues: Glu³¹ in FKBP12 is replaced by Gln³¹ from FKBP12.6 (E31Q); Asp³² in FKBP12 is replaced by Asn³² from FKBP12.6 (D32N); Trp⁵⁹ in FKBP12 is replaced by Phe⁵⁹ from FKBP12.6 (W59F). Mammalian FKBP12 and FKBP12.6 are highly conserved and Fig. S3 shows sequence alignment of several species demonstrating that the three residues that we chose to mutate were absolutely conserved in FKBP12 and were different but, again, absolutely conserved in FKBP12.6.

(FKBP12 purple; Protein Data Bank (PDB) 2DG3; FKBP12.6 green; PDB 1C9H) and illustrates their high structural similarity. Indeed, the root mean-squared deviation between the superimposed backbone atoms is only 0.44 Å. On the basis of their steric and electrostatic properties, we identified three residues that could have particular relevance. Glu³¹ and Asp³² are negatively charged residues of FKBP12, whereas the corresponding residues in FKBP12.6 are neutral (Gln³¹ and Asn³²). Trp⁵⁹ in FKBP12 is located within the hydrophobic binding pocket for rapamycin and has a larger side chain (an indole) compared with Phe⁵⁹ (a phenyl group) in FKBP12.6. The locations of these three amino acid residues in FKBP12 and the corresponding residues in FKBP12.6 are highlighted.

We produced a triple mutant of FKBP12 where these three highlighted amino acids were mutated to the corresponding residues in FKBP12.6 thus converting Glu³¹ to Gln, Asp³² to Asn, and Trp⁵⁹ to Phe. The affinity of various mutants of FKBP12 for RyR proteins have previously been investigated, (20,29) although the ability to influence RyR single-channel function (efficacy) has not been studied. The FKBP12_{E31Q/D32N/W59F} triple mutant retains affinity for RyR channels (20,29) and therefore is a molecule of choice for studying efficacy (because we must use mutant molecules that bind to RyR channels to study efficacy).

We then investigated the ability of FKBP12_{E31Q/D32N/W59F} to influence the gating of RyR1 and RyR2 under identical experimental conditions to those used with the wild-type proteins. Representative examples of the effects of FKBP12_{E31Q/D32N/W59F} on RyR2 gating are shown in Fig. 5. FKBP12_{E31Q/D32N/W59F} produced no observable changes in RyR2 gating even at nanomolar or micromolar concentrations. Clearly, the triple mutant protein, FKBP12_{E31Q/D32N/W59F}, has lost the normal ability of FKBP12 to activate RyR2 and behaves instead like FKBP12.6; efficacy has been lost.

When FKBP12_{E31Q/D32N/W59F} was added to RyR1 channels, an increase in P_o was observed (Fig. 6), very similar to that observed with FKBP12.6 (see Fig. 1). The representa-

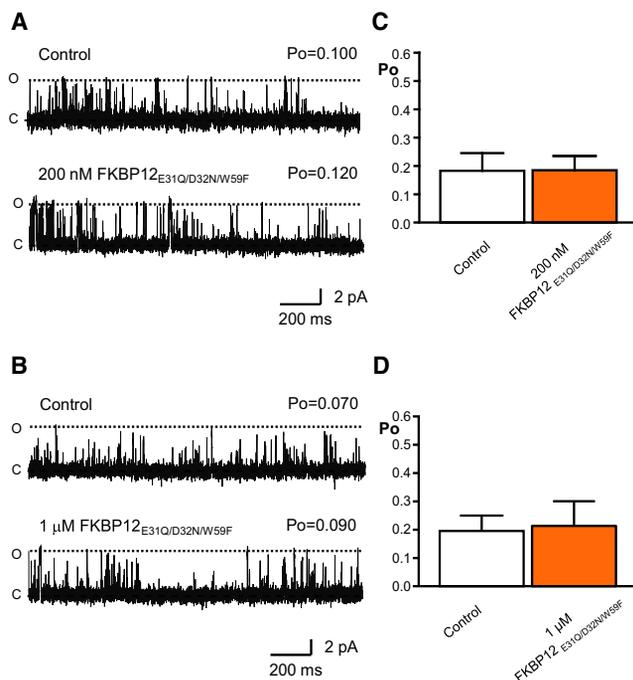


FIGURE 5 Effects of FKBP12_{E31Q/D32N/W59F} on sheep cardiac RyR2 gating. *A* and *B* are typical examples of single-channel experiments demonstrating that neither 200 nM (*A*) or 1 μ M (*B*) FKBP12_{E31Q/D32N/W59F} cause any observable effects on RyR2 activity. Dashed lines indicate open (O) and closed (C) channel levels, respectively. *C* and *D* illustrate mean P_o before and after addition of 200 nM (*C*) and 1 μ M (*D*) FKBP12_{E31Q/D32N/W59F}, respectively (SE; $n = 5$). To see this figure in color, go online.

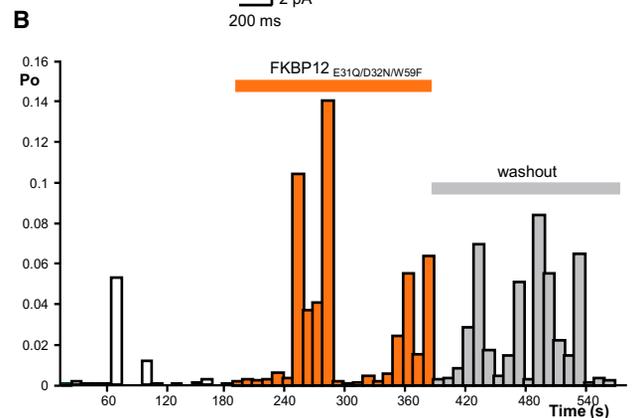
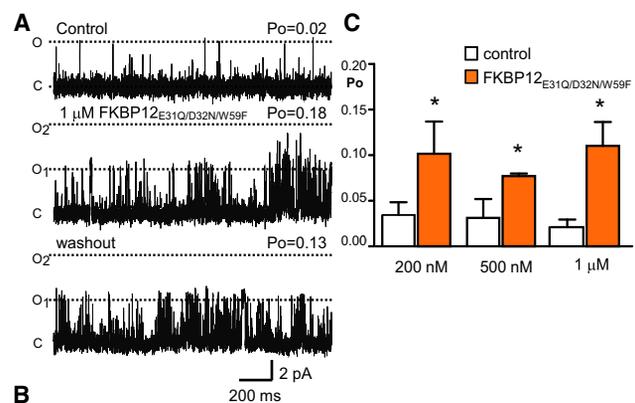


FIGURE 6 FKBP12_{E31Q/D32N/W59F} activates rabbit skeletal RyR1. (*A*) A typical experiment showing that 1 μ M FKBP12_{E31Q/D32N/W59F} activates RyR1. The effects were not reversed after washout of the *cis* chamber (*bottom* trace). Open (O₁, O₂) and closed (C) channel levels are marked with dashed lines. (*B*) Diary plot of a typical RyR1 single-channel experiment. After washout of the *cis* chamber, P_o did not reverse to control values. The bars indicate the incubation time with the mutant protein and subsequent washout of the protein from the *cis* chamber. (*C*) Mean P_o values before and after addition of 200 nM, 500 nM, and 1 μ M FKBP12_{E31Q/D32N/W59F} (SE; $n = 7-11$; $*p < 0.05$). Each concentration represents a set of independent experiments. To see this figure in color, go online.

typical experiment shown in Fig. 6 *A* illustrates the increase in the frequency of channel openings that was induced by FKBP12_{E31Q/D32N/W59F} and demonstrates that washout of the mutant protein from the cytosolic chamber could not reverse the effects of the mutant. To observe the variations in P_o that occur with time, diary plots of P_o against time were recorded (see Fig. 6 *B*). The effects of a range of concentrations of FKBP12_{E31Q/D32N/W59F} were investigated and in all cases, FKBP12_{E31Q/D32N/W59F} activated RyR1 (Fig. 6 *C*).

It is possible that some endogenous FKBP could still be associated with the channels in the bilayer before we add exogenous FKBP12. However, because we observe reproducible effects with both FKBP12 and FKBP12.6 and at both RyR1 and RyR2, it is obvious that there are always vacant FKBP binding sites on both channels that are functionally relevant. We do not know how many molecules of FKBP12 or FKBP12.6 must bind to RyR channels to produce their effect. The irreversible nature of the binding makes this difficult to examine at the single-channel level.

Many previous reports have used drugs such as rapamycin or FK-506 to strip FKBP from RyRs (9,11,30–33). However, even in these studies, it was not possible to be certain that all FKBP molecules were removed because Western blot was the only proof of FKBP dissociation and this is not a technique able to detect low levels of proteins. In fact, later work shows that these treatments do not achieve complete displacement of bound FKBP (34,35). We have therefore treated SR vesicles with rapamycin (20 μ M) using previously published methods (30) and performed mass spectrometry to detect the FKBP because this is a more sensitive method of protein detection than Western blot analysis. We find that FKBP12 is still detected with high confidence (false discovery rate < 1%; Table S1) demonstrating that rapamycin treatment does not remove all FKBP proteins from rabbit skeletal SR. Western blot analysis shown in Fig. 7, A and B, shows that rapamycin is very effective at dissociating FKBP from the SR but that there is a residual amount left which, depending on the

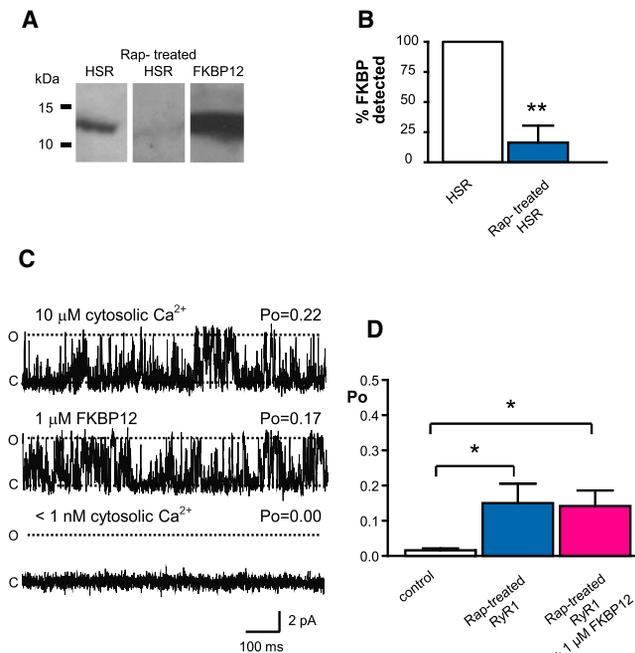


FIGURE 7 Rapamycin irreversibly activates rabbit skeletal RyR1. (A) Immunochemical detection of FKBP associated with skeletal HSR before (left lane) and after (middle lane) rapamycin treatment (as described in Methods). HSR was loaded at a protein concentration of 25 μ g. Recombinant purified FKBP12 was loaded at 500 ng as a positive control (right lane). (B) The percentage of FKBP detected in HSR after rapamycin treatment (Rap-treated HSR) is compared to control levels (HSR) (SD, $n = 3$, $**p < 0.01$). (C) Shows a representative example of a rapamycin pretreated RyR1 channel reconstituted into a bilayer. (O) and (C) indicate the open and the closed channel levels, respectively. Addition of 1 μ M FKBP12 to the cytosolic side of the channel (second trace) did not lower Po. Lowering the cytosolic $[Ca^{2+}]$ to <1 nM (by addition of 10 mM EGTA) shut the channel (bottom trace). (D) Mean Po values for control channels (untreated), rapamycin-treated channels (Rap-treated RyR1), and Rap-treated channels after addition of 1 μ M FKBP12 (Rap-treated RyR1 + 1 μ M FKBP12) are illustrated (SE; $n = 3$ –7; $*p < 0.05$). To see this figure in color, go online.

sensitivity of the antibody, may not always be immunodetectable. In the literature, there is heavy reliance on the ability of rapamycin to dissociate FKBP from RyR channels to infer mechanistic insight into FKBP effects on RyR function. However, such reasoning is questionable because there are reports that rapamycin may affect RyR channel behavior directly (35,36). We have therefore examined if rapamycin affects RyR1 gating by incorporating the rapamycin pretreated SR vesicles into bilayers. In line with the literature, we find that the Po of RyR1 channels pretreated with rapamycin is significantly higher than that of control channels (Fig. 7, C and D). Of importance, however, we find that addition of FKBP12 does not reduce Po values back to control levels (see second trace and Fig. 7 D) suggesting that the rapamycin-induced elevation of Po was not related to dissociation of FKBP12. The rapamycin-induced increase in Po was not caused by nonspecific damage to the channels because lowering cytosolic $[Ca^{2+}]$ to subactivating levels (third trace) completely closed the channels showing they were still sensitive to Ca^{2+} .

A rapamycin-induced increase in cardiac RyR2 Po has also been used to infer that dissociation of FKBP12.6 increases RyR2 Po (11) so we performed similar experiments with RyR2 (see Fig. S4). As with RyR1, we found that rapamycin increased RyR2 activity, although the effect was more marked. Washout of rapamycin did not lower Po, nor did the addition of FKBP12.6, although the channels retained sensitivity to cytosolic Ca^{2+} and were closed by reducing $[Ca^{2+}]$. Because rapamycin does not fully dissociate FKBP12 from RyR1 and because it irreversibly alters RyR1 and RyR2 channel gating, these experiments highlight the need to use native RyR channels that have not been chemically treated to probe the functional effects of FKBP12/FKBP12.6.

DISCUSSION

To our knowledge, a novel and unexpected finding of this study is that FKBP12.6 is a potent activator of the rabbit skeletal RyR1, whereas FKBP12 is an inhibitor. The results contrast with the situation in cardiac muscle where FKBP12 is an activator of RyR2 and FKBP12.6 is an antagonist of FKBP12 (15) but does not, itself, lower Po. We performed site-directed mutagenesis of the FKBP12 molecule and found that the selective ability of FKBP12 to activate RyR2 and inhibit RyR1 is contained within the three amino acid residues; Glu³¹, Asp³², and Trp⁵⁹. Mutation of these key residues to those found in FKBP12.6, caused a complete switch from FKBP12-like action to FKBP12.6-like action.

Experiments where sequential addition of FKBP12 and FKBP12.6 are made to the cytosolic face of RyR channels indicate that these proteins would compete for the same binding sites on RyR1 and RyR2. This is not surprising in view of the structural similarity of FKBP12 and FKBP12.6 but also in the light of cryo-electron microscopy

and single-particle three-dimensional reconstruction studies indicating that FKBP12 and FKBP12.6 would bind in the same locations on RyR1 and RyR2 (37–39). Use of fluorescence resonance energy transfer also suggests that FKBP12 and FKBP12.6 would bind in the same locations on RyR1 and RyR2 and with similar orientation (40).

We show that FKBP12 and FKBP12.6 exert different effects on the gating of both RyR1 and RyR2. This indicates that the binding sites on the RyR1 and RyR2 channel proteins could be composed of different amino acid residues. Confirmation of this idea comes from binding studies (12,28) using radiolabeled FKBP12/12.6, which indicate that canine cardiac and rabbit skeletal RyR have different affinity for FKBP12/12.6 (although it should be remembered that subsequent work demonstrated that canine RyR2 has unusually high affinity for FKBP12.6 that is not shared by other mammalian RyR2 isoforms (41)). Another explanation for the different functional effects of FKBP12/12.6 could be that the binding interactions between FKBP12/RyR1 and FKBP12/RyR2 produce different changes in channel gating simply because of subtle differences in RyR1 and RyR2 channel gating mechanisms. For example, RyR1 and RyR2 are regulated slightly differently by cytosolic Ca^{2+} , especially at inactivating $[Ca^{2+}]$ (42–44), and there are subtle differences in the mechanisms by which other ligands such as ATP, caffeine, or suramin activate RyR1 and RyR2 (45–48). We show that activation of RyR1 by FKBP12.6 involves an increase in the frequency of channel opening. This is similar to the mechanism by which cytosolic Ca^{2+} activates RyR1 (49) and therefore FKBP12.6 may be sensitizing the channel to cytosolic Ca^{2+} . We have previously demonstrated that FKBP12 sensitizes RyR2 to cytosolic Ca^{2+} (15) and therefore this may be a common mechanism by which FKBP12 regulate RyR channels. We cannot be so confident about the mechanism by which FKBP12 inhibits RyR1; it may reduce RyR1 sensitivity to cytosolic Ca^{2+} but because P_o is lowered so much, we cannot collect enough opening events for lifetime analysis. The important, take-home message, though, is that FKBP12 and FKBP12.6 drive P_o in opposite directions for both RyR1 and RyR2. This has important consequences for skeletal muscle function if the relative ability of FKBP12 and FKBP12.6 to bind to RyR1 is affected in stress, exercise, aging, or disease.

Although we and others have previously reported that FKBP12.6 does not, itself, reduce the P_o of RyR2 (12,14,28), opinion to the contrary still persists. Our experiments with rapamycin now indicate why this could be. Rapamycin is a frequently used tool for dissociating FKBP12 from RyR channels and irreversible increases in RyR P_o after rapamycin treatment have led to the conclusion that RyRs must be bound by FKBP12 to ensure stable low P_o channel gating (11,31,35,50). However, FKBP12 or FKBP12.6 was not added back in the bilayer experiments to examine if these proteins could reverse a rapamycin-

induced increase in P_o . We have now demonstrated that use of rapamycin may produce misleading results because it can irreversibly increase RyR P_o in a manner that is independent of the presence or absence of FKBP12. FK506 is another drug, used extensively to dissociate FKBP12 from RyRs. However, studies conducted on intact skeletal fibers have also shown effects of both rapamycin and FK506 on excitation-contraction coupling that are unrelated to the removal of FKBP12 (51,52). Again, incorrect conclusions could be drawn from using this compound if it was not completely removed during a subsequent experiment.

Another problem is that [3H]ryanodine binding has often been used to indicate RyR channel activity following rapamycin treatment rather than examining the detailed single-channel behavior of RyR1/RyR2 channels directly following incorporation into bilayers. [3H]ryanodine binding can provide an incorrect approximation of the effect of an intervention on RyR activity if that intervention also directly affects the binding of ryanodine. For example, calmodulin increases RyR2 P_o but decreases [3H]ryanodine binding because calmodulin also reduces the rate of association of ryanodine to RyR2 (53). There is no study that has investigated whether FKBP12 could directly alter the rate of association or dissociation of ryanodine to RyRs or, particularly pertinent to this study, whether rapamycin itself influences ryanodine binding independently of P_o . It is very relevant that a single-channel report by Ahern et al. (1997) (33) suggests that ryanodine modification of RyR1 may be reversible after treatment with FK506. Any such increase in the rate of dissociation of ryanodine from RyR channels would, naturally, affect the binding of [3H]ryanodine to cardiac or skeletal SR. We (and others (34,35)) have also shown that rapamycin-treated skeletal SR vesicles will still have residual FKBP12 molecules associated (even if below immunodetection levels) and this will be a significant confounding factor given the very high affinity of FKBP12 for RyR channels. Endogenous FKBP12 will be present within the incubation medium for the [3H]ryanodine binding assay and will already be bound to an unquantified number of RyR channels. Using [3H]ryanodine binding to skeletal SR vesicles to assay the functional actions of FKBP12 will therefore provide results that are difficult to interpret.

Our mutant experiments have begun to distinguish those regions of the FKBP12 proteins that are important for efficacy. The FKBP12 mutant, FKBP12_{E31Q/D32N/W59F}, clearly has efficacy as an activator of RyR1 but appears to have no or low efficacy as a regulator of RyR2. Our experiments do not provide accurate measurement of the affinity of FKBP12_{E31Q/D32N/W59F} for RyR1 or RyR2, however, they suggest that FKBP12_{E31Q/D32N/W59F} retains high affinity for both RyR1 and RyR2 because the effects of adding FKBP12_{E31Q/D32N/W59F} to the cytosolic chamber are irreversible after washout (see Fig. 6, A and C), and preaddition of FKBP12_{E31Q/D32N/W59F} prevents FKBP12 from activating RyR2 (data not shown). Indeed, this same triple FKBP12

mutant has previously been shown to be capable of displacing ^{35}S -labeled FKBP12 bound to skeletal and cardiac SR vesicles indicating that high affinity binding to RyR1 and RyR2 is retained (29). The FKBP12 single point mutants E31Q, D32N, and W59F also bind tightly to rabbit skeletal RyR1 as evidenced by coimmunoprecipitation of GST-mutant FKBP12 and RyR1 (20). Crystallographic data show that Trp⁵⁹ in FKBP12 and Phe⁵⁹ in FKBP12.6 are located within the rapamycin hydrophobic binding pocket. This region is also suggested to provide the hydrophobic cavity involved in binding RyR channels (37,38,54,55). Mutations at position 59 in FKBP12/FKBP12.6 clearly do not abolish the binding of FKBP12 to RyRs (20,29), although some change in affinity is indicated (29). The larger amino acid in FKBP12 does not alter the fold of the protein but it could influence the particular interactions between FKBP12 and RyR2 that lead to changes in channel gating.

The residues in position 31 and 32 point away from the hydrophobic core of the proteins, and there are no structural differences between FKBP12 and FKBP12.6 in this region, however these residues could be relevant for electrostatic reasons, as FKBP12 carries two negative charges very close to each other, which are not present in FKBP12.6. A recent publication (39) suggests involvement of electrostatic charges in the interactions between FKBP12 and RyR1 and the formation of a salt bridge between Asp³² on FKBP12 and Arg⁹⁷⁶ on RyR1, which stabilizes the binding. If this hypothesis is correct, the mutations E31Q and D32N (where two negative charges in FKBP12 are substituted by the corresponding neutral residues of FKBP12.6), could lead to a critical change in protein function as these residues may be crucial for producing the precise binding interactions that enable FKBP12 to inhibit RyR1 and activate RyR2.

Physiological and pathophysiological perspectives

Our study calls for a fresh evaluation of the roles of FKBP12 in cardiac and skeletal muscle and, in fact, in all tissues where RyR1 or RyR2 are present. It has generally been assumed that FKBP12 is the only relevant physiological regulator of RyR1, whereas FKBP12.6 is the only important isoform that influences the function of cardiac muscle and other tissues where RyR2 is expressed. Because our experiments clearly demonstrate that both isoforms of FKBP can modulate both isoforms of RyR at extremely low concentrations, and since both FKBP12 and FKBP12.6 are present in skeletal and cardiac cells (12,27,28), SR Ca^{2+} -release in cardiac and skeletal muscle may depend on the competitive, dual regulation by both FKBP12 and FKBP12.6. The literature reports levels of 1–3 μM FKBP12 in cardiac and skeletal muscle but much lower (100–200 nM (56)) or undetectable (12,27,28) levels of FKBP12.6. Regarding RyR2, it now appears that canine RyR2 is somewhat of an outlier in having particularly high affinity for FKBP12.6

(41). For all other species of RyR2 investigated, RyR2 appears to also have high affinity for FKBP12 (15,41). In sheep, mass spectrometry demonstrated that FKBP12 can be detected in membrane fractions containing RyR2 with high confidence, whereas FKBP12.6 can only be detected with low confidence (15). Recent immunoblot analysis also showed that FKBP12 is present in cardiac cells (and in membrane fractions containing RyR2) of three mammalian species (pig, rabbit, mouse) at much higher levels than FKBP12.6 (34). In skeletal muscle, there is evidence that FKBP12 is more likely to be associated with RyR1 than FKBP12.6 (34,57). However, because FKBP12.6 is present in skeletal muscle and because we show a functional effect of FKBP12.6 at very low concentrations, we conclude that FKBP12.6 may also play a role in regulating RyR1 in skeletal muscle.

It is clear that the relative level of expression of FKBP12 and FKBP12.6 is important. In this regard, it is notable that FKBP binding to RyRs has been reported to be reduced in certain skeletal muscle pathologies (2,3,5). This may reflect an altered ratio in the expression levels of FKBP12 and FKBP12.6 or could be due to changes to RyR1 that affect its relative affinity for FKBP12/FKBP12.6. For example, a reduction in RyR1/FKBP12 binding as reported for dystrophic muscle (5) and sarcopenia (58,59), would be expected to lead to increased RyR1 Po due to dissociation of FKBP12 (which reduces RyR1 Po) and the possible increased binding by FKBP12.6 (which increases RyR1 Po). Fig. 8 suggests how the dual regulation of SR Ca^{2+} -release by FKBP12 and FKBP12.6 may operate in skeletal muscle under physiological and pathophysiological conditions.

Our study has clear implications for other tissues where both RyRs and FKBP12 are expressed. We should not assume that FKBP12 will exclusively bind to RyR1 or that FKBP12.6 will only bind to RyR2, rather, that both proteins can interact with either RyR isoform and that the absolute binding stoichiometry may vary from tissue to tissue. There are many reports indicating that FKBP12 plays an important role in intracellular Ca^{2+} release in a wide range of different tissues and cell types. These include bladder smooth muscle (60), pancreatic β -cells (61), vascular smooth muscle (62), endothelial cells (63), and various neuronal cells (64). Changes to the relative FKBP12/FKBP12.6 expression level in these cells may have important pathological implications.

CONCLUSION

We have shown that RyR1 is activated by FKBP12.6 but inhibited by FKBP12 and use of the FKBP12_{E31Q/D32N/W59F} mutant demonstrates that efficacy is controlled by very small changes in FKBP structure. The experiments highlight the need to understand the functional consequences of FKBP12/FKBP12.6 binding to RyR channels at the single-channel level to recognize the putative physiological

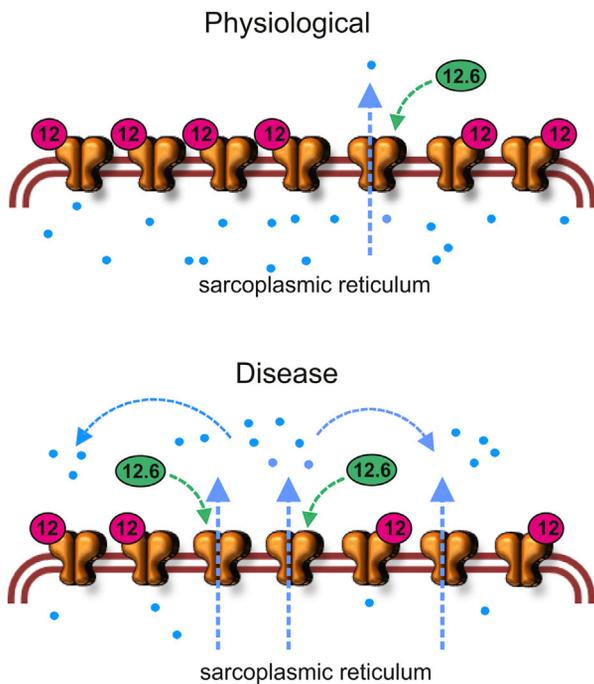


FIGURE 8 Proposed model of FKBP regulation of SR Ca^{2+} release in skeletal muscle. Under normal conditions, evidence suggests that RyR1 is predominantly bound by FKBP12, which maintains RyR1 in a low P_o mode. The occasional binding of FKBP12.6 to RyR1 would lead to a minority of channels with increased P_o . In disease or aging, it is reported that less FKBP12 is associated with RyR1. We speculate that a greater fraction of RyR1 channels may be bound by FKBP12.6, which would increase the numbers of leaky RyR1 channels. To see this figure in color, go online.

roles of FKBP/RyR interactions in different cell types and the changes that occur in disease and aging.

SUPPORTING MATERIAL

Four figures, one table, supporting data and reference (65) are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(14\)00061-7](http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00061-7).

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