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FKBP12 Binding Modulates Ryanodine Receptor Channel Gating*

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The ryanodine receptor (RyR1)/calcium release channel on the sarcoplasmic reticulum of skeletal muscle is comprised of four 565,000-dalton RyR1s, each of which binds one FK506 binding protein (FKBP12). RyR1 is required for excitation-contraction coupling in skeletal muscle. FKBP12, a *cis-trans* peptidyl-prolyl isomerase, is required for the normal gating of the RyR1 channel. In the absence of FKBP12, RyR1 channels exhibit increased gating frequency, suggesting that FKBP12 "stabilizes" the channel in the open and closed states. We now show that substitution of a Gly, Glu, or Ile for Val^{2461} in RyR1 prevents FKBP12 binding to RyR1, resulting in channels with increased gating frequency. In the case of the V2461I mutant RyR1, normal channel function can be restored by adding FKBP12.6, an isoform of FKBP12. These data identify Val^{2461} as a critical residue required for FKBP12 binding to RyR1 and demonstrate the functional role for FKBP12 in the RyR1 channel complex.

The ryanodine receptor (RyR)[1]-1/Ca^{2+} release channel on the sarcoplasmic reticulum of skeletal muscle is a tetrameric channel with a molecular mass of ~2.3 million daltons. The channel is comprised of a signaling complex that includes four molecules of FKBP12, originally identified as KC7, a peptide that co-purified with RyR1 (1), and kinases and phosphatases that regulate the channel function in particular by modulating the binding of the immunophilin subunit to the channel (2). FKBP12 is required for the normal gating of the channel and for coupled gating between neighboring channels (3, 4).

Binding of FKBP12 to RyR1 in skeletal muscle and the closely related isoform FKBP12.6 to RyR2 in cardiac muscle modulates channel gating. We have shown previously that dissociation of FKBP12 from RyR1 or FKBP12.6 from RyR2 channels increases the gating frequency of the channel manifested as reduced open and closed dwell times (2, 3, 5). Others have confirmed a role for FKBP12/12.6 in RyR1/RyR2 function (6–9), whereas one group has reported no functional role for FKBP12.6 in RyR2 (10). However, this same group has now reported that cardiomyocytes from an FKBP12.6 null mouse exhibit defects in Ca^{2+} signaling (11), consistent with a role for FKBP12.6 in regulating RyR2 as we showed originally (2, 5). Protein kinase A phosphorylation of RyR2 dissociates FKBP12.6 from the channel, resulting in increased Ca^{2+} sensitivity for activation (2). We have proposed that this is a physiological pathway (part of the "fight or flight" response) that regulates excitation-contraction coupling and specifically increases excitation-contraction coupling gain by increasing the amount of Ca^{2+} released for a given trigger (2, 12). In failing hearts this pathway is over-stimulated and becomes maladaptive, resulting in protein kinase A hyperphosphorylation of the RyR2 channels (2). Protein kinase A-hyperphosphorylated RyR2 channels in failing hearts are depleted of FKBP12.6 and exhibit single-channel properties (2) similar to those observed in RyR1 channels in the absence of FKBP12 (3).

A motif that can bind FKBP12 was suggested by studies that identified the ideal substrate for prolyl isomerization (13). In these studies Schreiber and co-workers (13) concluded that FKBP12 was binding to a twisted-amide transition state intermediate in the peptidyl-prolyl bond of its substrates. They subsequently showed that FK506 and rapamycin, immunosuppressant drugs that bind to FKBP12, are twisted amide surrogates. The binding of FKBP12 to the hydrophobic motif identified as the optimal substrate for peptidyl-prolyl isomerization present in the inositol 1,4,5-triphosphate receptor (an intracellular Ca^{2+} release channel with structural homology to RyR channels) was examined subsequently using a yeast two-hybrid interaction screen (14). The present study is the first to establish the precise location of an FKBP12 binding site on the intact full-length RyR1 channel. We show that Val^{2461} is a key residue mediating FKBP12 binding to RyR1 and that mutations at this site that inhibit FKBP12 binding to RyR1 result in significant defects in single-channel function, which is consistent with altered channel gating because of the absence of FKBP12. To demonstrate that the defects in single-channel properties are caused by lack of FKBP12 binding as opposed to some other structural change in the channel, we generated a mutant V2461I RyR1 channel that does not bind FKBP12 but does bind FKBP12.6. Addition of FKBP12.6 to the V2461I mutant RyR1 restores normal channel function.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Wild-type and Mutant RyR1—Mutagenesis of RyR1 was performed using a cassette containing nucleotides 6597–11766. The sequences of the primers used for mutagenesis (including conservative substitutions to generate new restriction sites) were as follows: V2461H, c cgc gcc atc ctt cgc agt ctt cat ccc ctg gac gac c; V2461E, c ctt cgc tcc ctc gag ccc ctg gac gac c; V2461G, c ctt cgc tcc ctc ggg ccc ctg gac gac c; and V2461I, cc atc ctt cgc tca tta att ccc ctg gac gac c.

The Morph site-specific plasmid DNA mutagenesis kit (5 Prime → 3 Prime, Boulder, CO) was used according to the manufacturer's instructions. The corresponding mutant fragments were digested with *Nsi*I

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[1] The abbreviations used are: RyR, ryanodine receptor; FKBP12, FK506-binding protein.

and *Sap*I (at nucleotides 7453 and 7647, respectively) and subcloned into an expression vector (pCMV5) containing a full-length RyR1 clone cut with the same enzymes. HEK293 cells were maintained in T175 flasks in minimum Eagle's media containing 10% fetal bovine serum and were passed every 3–4 days. One T175 flask (50% confluent) was transfected with 20.0 μg of DNA using the Ca^{2+} phosphate precipitation method. Forty-eight h post-transfection, the cells were washed twice, scraped into phosphate-buffered saline, and pelleted by centrifugation at 2500 × *g* for 5 min at 4 °C. After resuspending the pellet in 0.5 ml of 20 mM HEPES-NaOH, pH 7.5, containing protease inhibitors (complete EDTA-free inhibitors from Roche Molecular Biochemicals), the cells were allowed to swell for 30 min on ice before lysis by 20 strokes of a Dounce homogenizer. Cell homogenates were diluted with an equal volume of ice-cold medium containing 500 mM sucrose and 10 mM HEPES, pH 7.2, and centrifuged at 10,000 × *g* for 15 min. Supernatants were recovered and centrifuged at 100,000 × *g* for 45 min. Pellets were resuspended in a buffer containing 250 mM sucrose, and the protein concentration of the microsomes was determined by Bradford assay. Aliquots were stored at −80 °C.

Immunoprecipitations—Approximately 200 μg of wild-type or mutant RyR1 containing microsomes were diluted to a total of 500 μl with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 0.25% sodium deoxycholate, 150 mM NaCl, 1.0 mM EDTA, 1.0 mM NaF, 1.0 mM Na_3VO_4, and 0.25% Triton X-100) containing protease inhibitors. These samples were incubated with 2.0 μl of anti-RyR antibody for 1 h at 4 °C followed by the addition of 40 μl of protein A-Sepharose (1:1 in phosphate-buffered saline) for 1 h at 4 °C. After the beads were washed four times for 5 min in immunoprecipitation buffer, the proteins were eluted with SDS sample buffer, heated to 95 °C for 4 min, and assayed for both RyR and FKBP by Western analysis.

Stripping FKBP12 from RyR1 and Rebinding FKBP12.6—Recombinant RyR1 microsomes were washed in 0.5 ml of 10 mM HEPES-NaOH, pH 7.5, containing protease inhibitors and were centrifuged at 100,000 × *g* for 45 min. Pellets were resuspended in a buffer containing 10 mM HEPES, 300 mM sucrose, and 0.9% NaCl. Microsomes (200 μg) were added to 0.2 ml of imidazole buffer (5 mM imidazole, pH 7.4, and 0.3 M sucrose) and incubated for 1 h at 37 °C with varying concentrations of rapamycin. Samples were centrifuged at 95,000 × *g* for 10 min, and the supernatants were collected. Pellets were washed two times in 0.2 ml of imidazole buffer and centrifuged at 95,000 × *g* for 10 min. The final pellet was resuspended in 0.2 ml of imidazole buffer. Both the pellet and supernatant were separated by polyacrylamide gel electrophoresis and analyzed for FKBP12 by Western blotting.

Recombinant RyR1 containing microsomes was resuspended in 250 μl of imidazole buffer (5 mM imidazole, pH 7.4, and 0.3 M sucrose) and incubated with 5 μM recombinant FKBP12.6 for 4 h at 4 °C. Samples were centrifuged at 95,000 × *g* for 10 min, and the pellets were washed twice and resuspended in 500 ml of immunoprecipitation buffer. RyR1 was immunoprecipitated from these samples as described above.

Single-channel Recordings—Single-channel experiments were conducted under voltage-clamp conditions. Sarcoplasmic reticulum vesicles were added to the *cis* chamber and induced to fuse with planar lipid bilayers composed from 3:1 phosphatidyl ethanolamine/phosphatidyl serine (Avanti Polar Lipids, Birmingham, AL). The bilayer cup was made of polystyrene with a 0.15-mm aperture. Fusion was promoted by KCl added to the *cis* chamber. After incorporation of a single Ca^{2+} release channel, the KCl gradient was eliminated by perfusion of the *cis* chamber with *cis* solution. Solutions used for channel analysis were as follows: *trans* solution, 250 mM Hepes, 53 mM $Ca(OH)_2$, 50 mM KCl, pH 7.35; and *cis* solution, 250 mM HEPES, 125 mM Tris, 50 mM KCl, 1 mM EGTA, 0.5 mM $CaCl_2$, pH 7.35. Free Ca^{2+} concentration was determined using the program CHELATOR (15). The *trans* chamber was connected to the head-stage input of an Axon 200 amplifier (Axon Instruments) using a silver/AgCl electrode and agar/KCl bridge. The *cis* chamber was held at ground with a similar electrode. The single-channel currents were filtered at 1 kHz with an 8-pole Bessel filter (Warner Instruments) and digitized at 4 kHz. Data were collected on a Pentium computer using AxoScope1 (Axon Instruments) and a Digidata 1200 (Axon Instruments) interface. The pClamp 6 program (Axon Instruments) was used for analyzing single-channel data. Open probability and the lifetime of open events and gating frequency were identified by 50% threshold analysis using at least 2 min of continuous record. The Student's *t* test was used for statistical analyses.

RESULTS

To identify determinants for the binding of FKBP12 to the RyR1 channel we generated four mutant forms of the 5037-amino acid RyR1 channel subunit. In each case we introduced substitutions for Val^{2461}, which is in the immediately N-terminal position relative to Pro^{2462} (referred to as the P1 position) predicted to form a binding site for FKBP12 on RyR1 (2, 14). The choice of amino acids to substitute for Val^{2461} was guided by data showing that the P1 amino acid strongly influences the catalytic efficiency for *cis-trans* peptidyl-prolyl isomerization by FKBP12 of synthetic peptide substrates (13). Hydrophobic residues (Leu, Ile, Phe, and Val) in the P1 position resulted in the highest catalytic efficiency, whereas charged residues (His and Glu) reduced the catalytic efficiency, and Gly exhibited the lowest catalytic efficiency (13).

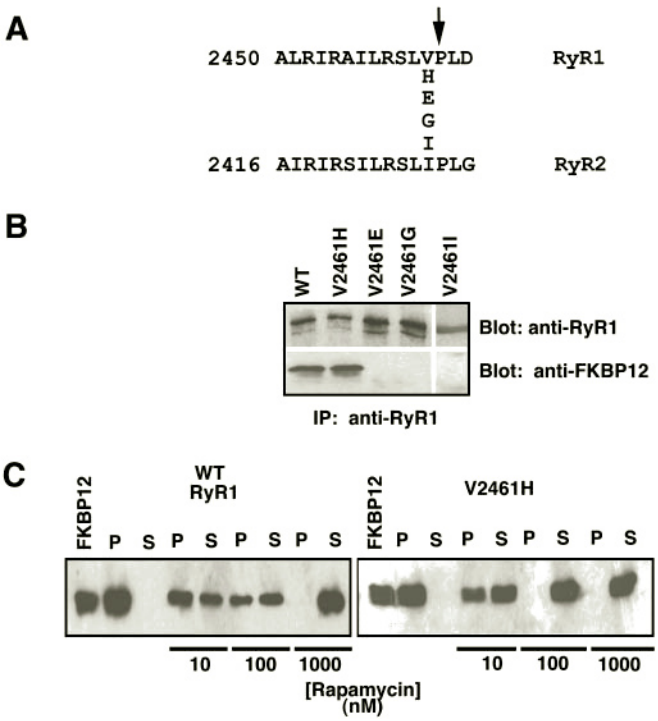

FIG. 1. **Mutations in RyR1 eliminate FKBP12 binding to the channel.** *A*, four mutant RyR1 channels were generated, each with a single amino acid substitution in the P1 position relative to Pro^{2462} (denoted by the *arrow*). FKBP12/12.6 binding sites are compared for RyR1 and RyR2. *B*, co-immunoprecipitations of RyR1 and FKBP12 were performed as described previously (16), showing that FKBP12 co-immunoprecipitated with wild-type (*WT*) RyR1 and with the V2461H mutant but not with the V2461G, V2461E, or V2461I mutant channels. *C*, FKBP12 was competed off from wild-type and mutant V2461H RyR1 with rapamycin. Microsomes containing recombinant RyR1 were incubated with varying concentrations of rapamycin as shown, and RyR1 was sedimented by centrifugation followed by immunoblotting the pellet (*P*) and supernatant (*S*) using the anti-FKBP12 antibody.

Therefore we generated four mutant forms of RyR1, V2461G, V2461E, V2461H, and V2461I (Fig. 1*A*). Wild-type and mutant RyR1 were expressed in HEK293 cells (which contain endogenous FKBP12). Co-immunoprecipitation experiments were performed to determine whether FKBP12 was associated physically with the recombinant wild-type and mutant RyR1 as we had demonstrated previously for native RyR1 (16) and for recombinant RyR1 expressed in Sf9 cells (3). FKBP12 co-immunoprecipitated with the recombinant wild-type RyR1 and with the V2461H mutant but not with the V2461G, V2461E, or V2461I mutant forms of RyR1 (Fig. 1*B*). These data identify the P1 residue Val^{2461} as being critical in determining the binding of FKBP12 to RyR1.

FKBP12 can be competed off from RyR1 using the immunosuppressant drugs FK506 or rapamycin at nanomolar concentrations (3). Experiments were performed in which membranes containing recombinant wild-type or mutant RyR1 were iso-

TABLE I
Single channel properties of wild-type and mutant recombinant RyR1
All values are expressed as mean ± S.D. Channels were recorded using $[Ca^{2+}]_{cis}$ = 150 nM.

	Number of experiments	Open probability	Gating frequency	Open time
			s^{-1}	*ms*
Wild-type	11	0.014 ± 0.022	0.72 ± 0.83	10.68 ± 15.54
V2461H	9	0.018 ± 0.013	1.33 ± 1.31	13.21 ± 10.03
V2461G	16	0.080 ± 0.065[a]	23.02 ± 13.76[b]	2.31 ± 1.09[a]
V2461E	21	0.025 ± 0.030	19.06 ± 20.57[a]	1.27 ± 0.49[b]
V2461I	22	0.032 ± 0.055	13.21 ± 13.89[a]	1.51 ± 0.97[b]
V2461I + FKBP12	8	0.013 ± 0.013	7.41 ± 6.73[b]	1.44 ± 0.37[a]
V2461I + FKBP12.6	12	0.005 ± 0.005	0.33 ± 0.30	12.64 ± 7.84

[a] $p \leq 0.05$ compared to wild-type channels.
[b] $p \leq 0.01$ compared to wild-type channels.

lated from transfected HEK293 cells, centrifuged, and assayed by SDS-polyacrylamide gel electrophoresis/immunoblotting to determine whether FKBP12 is bound to RyR1 (in the pellet, *P*, Fig. 1*C*). Treatment of the membranes with rapamycin dissociates RyR1-bound FKBP12 so that it appears in the supernatant, *S*. Compared with wild type RyR1, rapamycin sensitivity of the V2461H mutant channel was increased ~10-fold. Rapamycin competed FKBP12 off from the V2461H mutant channel at a concentration of 10 nM, and complete removal of FKBP12 was achieved with 100 nM compared with 1000 nM for the wild-type channel (Fig. 1*C*). The V2461E, V2461G, and V2461I mutant RyR1 channels did not bind FKBP12 in this assay (data not shown), which is in agreement with the co-immunoprecipitation experiments.

To analyze the functional consequences of reduced affinity for FKBP12 in the mutant RyR1 channels, the single-channel properties of recombinant proteins were examined in planar lipid bilayers (Table I). One of the prominent features of RyR1 expressed in the absence of FKBP12 is an increase in the gating frequency compared with native RyR1 or wild-type RyR1 channels co-expressed with FKBP12 (3). The wild-type recombinant RyR1 channel expressed in HEK293 cells (which contain endogenous FKBP12) exhibited the same single-channel properties (n = 11, *e.g.* Fig. 2*A* and Table I) as reported previously for native RyR1 and for wild-type RyR1 co-expressed with FKBP12 in Sf9 cells (3, 17). The V2461H mutant RyR1 channel that binds FKBP12 but with reduced affinity (Fig. 1*C*) also exhibited normal single-channel properties (n = 9, *e.g.* Fig. 2*B* and Table I). However, the V2461E mutant RyR1 channel did exhibit a significant increase in the gating frequency (n = 21, *e.g.* Fig. 2*C* and Table I) as did the V2461G mutant RyR1 (n = 16, *e.g.* Fig. 2*D* and Table I). This rank order of the appearance of increased gating frequency in the mutant RyR1 channels correlates with the increased affinity of FKBP12 for substrates with Val in the P1 position compared with those with His, Glu, or Gly. The amplitude histograms for the V2461E (Fig. 2*C*), V2461G (Fig. 2*D*), and V2461I (Fig. 4*C*) mutant RyR1 channels suggest that these mutant channels exhibit partial openings or subconductance states as reported previously for RyR1 expressed in the absence of FKBP12 (3). However, given the increased gating frequency of these mutant channels and the time resolution of our recording system (~1 ms), we are not able to resolve the five discrete states (closed, 1/4, 1/2, 3/4, fully open) reported previously (3).

The open dwell times, gating frequency, and open probability of the wild-type RyR1 and mutant V2461H channels were similar (Table I). In contrast, the open dwell times were significantly shorter, and the gating frequency was significantly higher for the V2461E and V2461G mutant channels compared with the wild-type channel (Table I). These data indicate that the V2461E and V2461G mutant channels are less stable in the open or closed states as suggested previously for RyR1 channels expressed in the absence of FKBP12 (3).

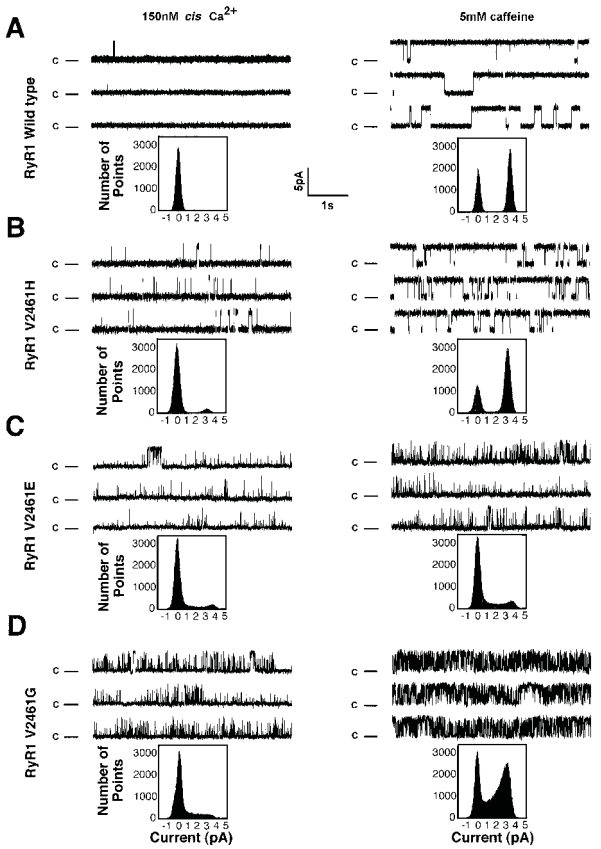

FIG. 2. **Altered single-channel properties of mutant RyR1 channels that do not bind FKBP12.** Characterization of recombinant wild type (*A*) and mutant V2461H (*B*), V2461E (*C*), and V2461G (*D*) RyR1 channels is shown. Representative current traces under control conditions *cis* (cytosolic) Ca^{2+} 150 nM and after the addition of caffeine (5 mM) are shown also. Single-channel currents were recorded at 0 mV (*trans versus cis*) in asymmetrical Ca^{2+} solutions (*cis* = 150 nM Ca^{2+} free, *trans* = 53 mM $Ca(OH)_2$). All-points amplitude histograms were generated from 5×10^4 points. *c* indicates the closed state of the channels. Channel openings are upward.

One possible explanation for the observed changes in single-channel function in the RyR1 mutants that do not bind FKBP12 is that the substituted amino acids cause structural changes in the channel that result in altered function independent of FKBP12 binding. To address this possibility specifically we designed a mutant RyR1 (V2461I) that does not bind FKBP12 but does bind FKBP12.6 and therefore could be "rescued" by the addition of FKBP12.6. The ability to restore normal function to the mutant RyR1 channel would exclude the possibility that the amino acid substitutions themselves cause

the observed changes in channel function independent of FKBP12 binding. Using co-immunoprecipitations, we showed that the V2461I mutant RyR1 (expressed in HEK293 cells that contain FKBP12 but not FKBP12.6) binds FKBP12.6 (Fig. 3) but not FKBP12 (Fig. 1*B*). When the V2461I mutant channel was examined in planar lipid bilayers, it exhibited the same increased gating frequency as the V2461G mutant RyR1 (Fig. 4*B*). However, when FKBP12.6 was added to the RyR1 V2461I mutant, normal channel function comparable with that of the wild-type RyR1 was restored (Fig. 4*D*). In contrast, the addition of FKBP12 to the V2461I mutant RyR1 channel failed to restore normal channel function (Fig. 4*C*).

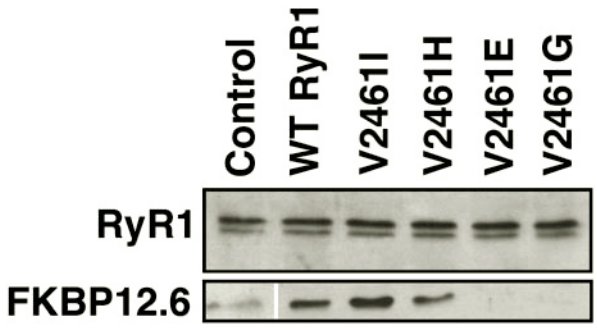

FIG. 3. **Some mutant RyR1 channels that do not bind FKBP12 do bind FKBP12.6.** Wild-type and mutant RyR1 were incubated with recombinant FKBP12.6. Co-immunoprecipitations of RyR1 and FKBP12.6 were performed as described previously (2), showing that FKBP12.6 co-immunoprecipitated with wild type (*WT*) RyR1 and with the V2461I and V2461H mutants but not with the V2461G or V2461E mutant RyR1 channels. *Control*, RyR1 from skeletal muscle and recombinant FKBP12.6.

DISCUSSION

The mechanism whereby FKBP12 catalyzes the *cis-trans* isomerization of peptidyl-prolyl bonds is to bind to and lower the energy of the unstable twisted-amide transition state intermediate (18). FKBP12 also may bind to the twisted-amide transition state intermediate of a peptidyl-prolyl bond on RyR1. Stabilization of an unstable bond may be part of the mechanism whereby binding of FKBP12 allows the RyR1 channel to assume a conformation that permits optimal gating (3) as well as coupled gating between neighboring channels (4). As such, this would be a novel role for an immunophilin and would imply that steric constraints hinder the complete isomerization of the peptidyl-prolyl bond to either fully *cis* or *trans* conformations. This is because the affinity of FKBP12 for either the *cis* or *trans* conformation of the peptidyl-prolyl bond would be reduced, whereas evidence suggests that *in vivo* FKBP12 is bound tightly to RyR1. Because the interaction between FKBP12 and RyR1 likely involves the catalytic site of the enzyme, tight *in vivo* binding to the substrate would not be

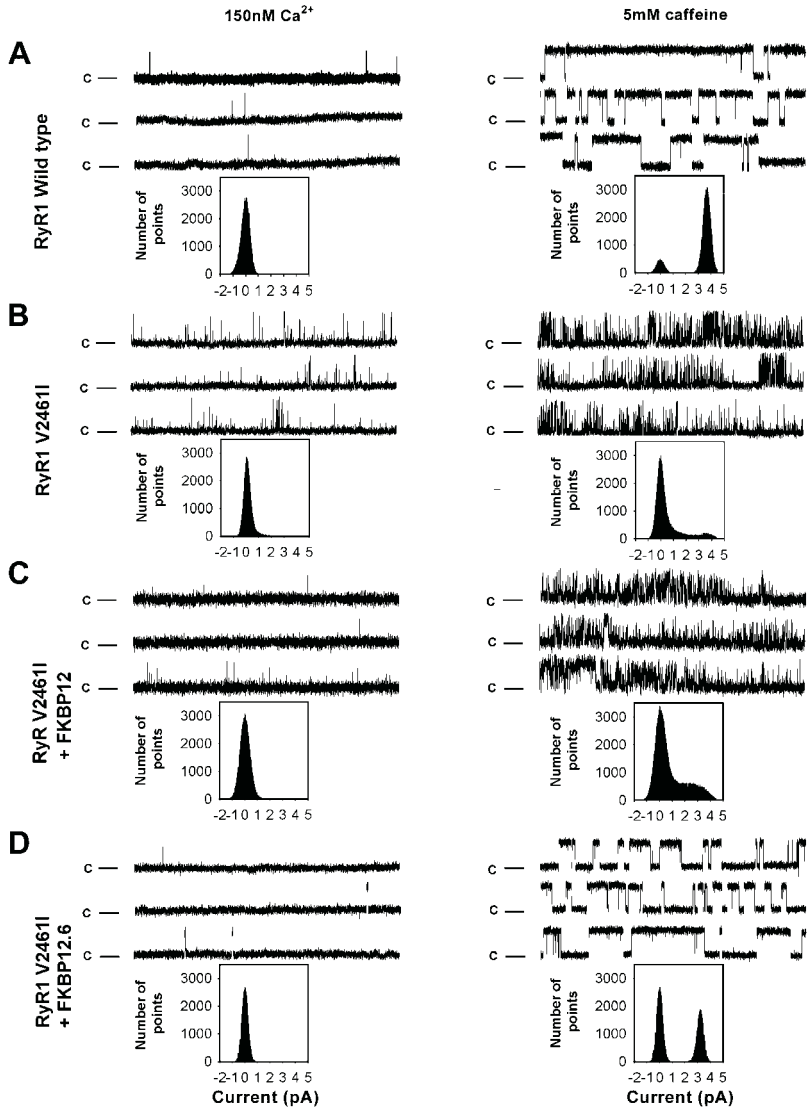

FIG. 4. **FKBP12.6 (but not FKBP12) restores normal channel function to the V2461I mutant RyR1 channel.** Characterization of recombinant wild type (*A*) and mutant V2461I (*B*), V2461I plus FKBP12 (*C*), and V2461I plus FKBP12.6 (*D*) RyR1 channels is shown. Representative current traces under control conditions *cis* (cytosolic) Ca^{2+} 150 nM and after the addition of caffeine (5 mM) are shown also. Single-channel currents were recorded at 0 mV (*trans versus cis*) in asymmetrical Ca^{2+} solutions (*cis* = 150 nM Ca^{2+} free, *trans* = 53 mM $Ca(OH)_2$). All-points amplitude histograms were generated from 5×10^4 points. *c* indicates the closed state of the channels. Channel openings are upward.

consistent with RyR1-bound FKBP12 functioning as a true enzyme.

The results for the binding of FKBP12 to V2461H, V2461E, and V2461G mutant RyR1 channels correlate precisely with the increased affinity of FKBP12 for substrates with a hydrophobic residue at the P1 position Gly (13); however, the lack of FKBP12 binding to the V2461I mutant RyR1 diverges from this model. Interestingly, the catalytic efficiency (k_{cat}/K_m) of FKBP12-catalyzed reactions for peptide substrates in which the amino acid residue at the P1 position is varied is 7-fold higher for substrates with Val (wild type) at the P1 position compared with His, 37-fold higher compared with Glu, and 41-fold higher compared with Gly (13). This rank order of efficiency of the FKBP12-catalyzed isomerization of peptide substrates has been used to support the idea that stabilization of the twisted-amide intermediate is the mechanism of amide rotation by FKBP12 (18). Because the rank order of the catalytic efficiency of FKBP12 correlates with the binding of FKBP12 to the wild-type and three of the four mutant RyR1 channels FKBP12 (Fig. 1), the mechanism by which FKBP12 modulates RyR1 channel gating may involve binding to and stabilization of a twisted-amide transition state intermediate of a peptidyl-prolyl bond in RyR1. However, the finding that FKBP12 does not bind to the V2461I mutant RyR1 would seem to cast some doubt on this interpretation.

Compared with peptides with Gly at the P1 position, the catalytic efficiency of FKBP12-catalyzed reactions was 3-fold higher when the P1 residue was a Leu and 2-fold higher when the P1 residue was an Ile (13). Among the other channels and receptors known to bind FKBP12, three contain a Leu (inositol 1,4,5-triphosphate receptors 1 and 2 and TβRI) at the P1 position and one (RyR2) has an Ile at the P1 position. The finding that the V2461I mutant RyR1 channel does not bind FKBP12 was not predicted on the basis of examining the catalytic efficiency of FKBP12 because the enzyme isomerizes a synthetic peptide with Ile in the P1 position more efficiently than one with Val (13). Our data show that FKBP12.6 binds with high affinity to the V2461I mutant RyR1 just as it binds to the wild-type RyR2 channel that has an Ile in the corresponding P1 position (Fig. 1*A*). The binding of FKBP12.6 but not FKBP12 to the V2461I RyR1 mutant could be explained if the catalytic sites formed by hydrophobic pockets were slightly different in size such that FKBP12.6 accepted the side chain of an Ile, whereas FKBP12 does not. An alternative explanation would be that it is not the catalytic site of FKBP12/12.6 that binds to the residue at position 2461 in RyR1, leaving open the possibility that the catalytic site is interacting with another peptidyl-prolyl bond in the channel.

The structure of FKBP12 bound to the type 1 transforming growth factor β receptor shows that the P1 residue and the Pro in the FKBP12 binding site do not interact with FKBP12. However, this report also notes that there are other contact sites between FKBP12 and transforming growth factor β receptor (19). The data in the present study suggest that the FKBP12 binding site on RyR1 may have a significantly different structure from that on the transforming growth factor β receptor because mutations of the P1 residue strongly influence binding of FKBP12 but also does not exclude the possibility that other residues in RyR1 (and by inference in RyR2) besides the P1 residue participate in FKBP12/12.6 binding to the channels. Rather, the present study establishes the critical role of Val^{2461} in FKBP12 binding.

Single-channel data showing defects in mutant RyR1 channels that do not bind FKBP12 are consistent with data from RyR1 channels expressed in Sf9 cells that lack FKBP12 (3) and RyR1 channels from the FKBP12 knockout mouse that exhibited similar single-channel defects (20).

In addition to the effects on single-channel properties noted above, a role for FKBP12 in coupled gating between neighboring RyR1 channels has been proposed (4). One model for how FKBP12 can influence gating of individual channels is that each of the subunits of RyR1 contribute a pore through the sarcoplasmic reticulum membrane, and FKBP12 stabilizes the channel in a conformation that permits the four subunits of the channel to gate simultaneously (21). To explain how FKBP12 can also influence coupled gating between channels, the model would have to include stabilization of a conformational state that enhances interactions between neighboring channels (21).

The present study supports the concept that FKBP12 binding to RyR1 stabilizes the channel in a favorable conformation. As such, this is a novel mechanism for regulating ion channel gating. The substitution of Gly for Val^{2461} at the P1 position would be expected to introduce significantly increased flexibility around the peptidyl-prolyl bond at this position because of the small size of Gly. Increased flexibility around the Gly-Pro bond might destabilize the channel contributing to the reduction in dwell times and the corresponding increase in gating frequency exhibited by the V2461G mutant RyR1 channels. The demonstration that FKBP12.6 can restore normal function to the V2461I mutant channel excludes the possibility that the observed defects in single-channel function are caused by the amino acid substitution independent of whether FKBP12 is bound to the channel.

Taken together our data indicate that Val^{2461} is a critical determinant of FKBP12 binding to RyR1, which is required for normal channel function. However, these data do not exclude the possibility that there are other contact sites between FKBP12 and RyR1 and other residues in RyR1 that also are required for binding of FKBP12 to the channel.

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