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FKBP12 Binding to RyR1 Modulates Excitation-Contraction Coupling in Mouse Skeletal Myotubes*

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The skeletal muscle sarcoplasmic reticulum (SR) Ca^{2+} release channel or ryanodine receptor (RyR1) binds four molecules of FKBP12, and the interaction of FKBP12 with RyR1 regulates both unitary and coupled gating of the channel. We have characterized the physiologic effects of previously identified mutations in RyR1 that disrupt FKBP12 binding (V2461G and V2461I) on excitation-contraction (EC) coupling and intracellular Ca^{2+} homeostasis following their expression in skeletal myotubes derived from RyR1-knockout (dyspedic) mice. Wild-type RyR1-, V246I-, and V2461G-expressing myotubes exhibited similar resting Ca^{2+} levels and maximal responses to caffeine (10 mM) and cyclopiazonic acid (30 μM). However, maximal voltage-gated Ca^{2+} release in V2461G-expressing myotubes was reduced by ~50% compared with that attributable to wild-type RyR1 ($\Delta F/F_{max}$ = 1.6 ± 0.2 and 3.1 ± 0.4, respectively). Dyspedic myotubes expressing the V2461I mutant protein, that binds FKBP12.6 but not FKBP12, exhibited a comparable reduction in voltage-gated SR Ca^{2+} release ($\Delta F/F_{max}$ = 1.0 ± 0.1). However, voltage-gated Ca^{2+} release in V2461I-expressing myotubes was restored to a normal level ($\Delta F/F_{max}$ = 2.9 ± 0.6) following co-expression of FKBP12.6. None of the mutations that disrupted FKBP binding to RyR1 significantly affected RyR1-mediated enhancement of L-type Ca^{2+} channel activity (retrograde coupling). These data demonstrate that FKBP12 binding to RyR1 enhances the gain of skeletal muscle EC coupling.

Depolarization of skeletal muscle activates sarcolemmal voltage sensors that trigger the opening of nearby intracellular Ca^{2+} release channels located in the sarcoplasmic reticulum (SR),[1] a process termed excitation-contraction (EC) coupling (1). Homotetramers of the skeletal muscle isoform of the ryanodine receptor (RyR1) function as SR Ca^{2+} release channels, and the dihydropyridine receptor (DHPR) functions both as the sarcolemmal voltage sensor for skeletal muscle EC coupling and as an L-type Ca^{2+} channel (L-channel). The coupling between DHPR and RyR1 protein complexes in skeletal muscle is thought to involve a mechanical, and presumably physical, interaction (1). In fact, DHPR-RyR1 mechanical coupling represents a bi-directional signaling interaction since DHPRs control the gating of SR Ca^{2+} release channels (orthograde coupling), and the presence of RyR1 enhances the Ca^{2+} conducting activity and modifies the gating properties of the skeletal L-channel (retrograde coupling) (2–4).

SR Ca^{2+} release channels in skeletal muscle are composed of four identical RyR1 protomers, each of which is tightly associated with a single 12-kDa FK506-binding protein (FKBP12) (5). FKBP12 is known to physically associate with RyR1 based on the fact that they are co-purified on linear sucrose gradients, RyR1 is co-immunoprecipitated from crude muscle homogenates by FKBP12 antibodies, both proteins co-localize during immunocytochemistry (6), and they are found to be associated using electron cryomicroscopy and three-dimensional reconstruction (7). In three-dimensional reconstructions of the release channel, FKBP12 molecules bind to the surface of the RyR that opposes the t-tubule membrane, adjacent to each of the four separate "clamp" regions of the release channel (7). Despite adjacent positioning of FKBP12 and the DHPR at the clamp regions of RyR1, the precise role of FKBP12 in modulating skeletal muscle EC coupling has yet to be elucidated. Nevertheless, it is tempting to speculate that the clamp regions represent a control point for skeletal muscle EC coupling and that this control is exerted by complex interactions between the DHPR, RyR1, and FKBP12.

The binding of FKBP12 to RyR1 profoundly alters the functional properties of SR Ca^{2+} release channels incorporated into planar lipid bilayers (8–13). These studies have clearly shown that FKBP12 coordinates cooperative gating of the four RyR1 subunits, eliminating subconductance states and preferentially stabilizing the fully open and closed states of the channel. FKBP-depleted release channels exhibit longer mean open times, have a greater open probability, and show an increased prevalence of substate activity (8–10, 12). Moreover, FKBP12 enhances cooperative gating between neighboring homotetramers, resulting in the "coupled gating" of adjacent release channels (11) and also regulates ryanodine receptor adaptation (13). Interestingly, recent results indicate that peptides derived from the skeletal L-channel II-III loop directly activate Ca^{2+} release channels and that this activation is substantially reduced for FKBP12-stripped channels (14, 15). Taken together, these results suggest that the FKBP12-RyR1 interaction alters

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; L-channel, L-type Ca^{2+} channel; EC, excitation-contraction; RyRs, ryanodine receptors; CICR, Ca^{2+}-induced Ca^{2+}-release; FKBP, FK-506-binding protein; IP_3, inositol 1,4,5-trisphosphate.

the functional coupling between the DHPR and RyR1 protein complexes. However, the precise role of FKBP12 in modulating EC coupling in intact skeletal muscle remains controversial. For example, immunosuppressant drugs such as rapamycin and FK506 disrupt the FKBP12-RyR1 interaction and cause a loss of depolarization-induced contractile ability in rat muscle fibers (16). In contrast, the observation that FKBP12-knockout mice fail to exhibit gross morphological or functional skeletal muscle abnormalities casts doubt on suggestions that FKBP12 plays an essential role in skeletal muscle EC coupling (17).

The RyR1 amino acids that comprise the FKBP binding site have yet to be definitively mapped. However, FKBP12 has been proposed to bind to the type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1) at a conserved proline-containing hydrophobic di-peptide epitope (LP, VP, or IP), which is found in all known IP_3R and RyR isoforms. Mutating the conserved proline residue to a glutamine abolishes FKBP12 binding to IP_3R1 (18). However, despite the conservation of the proline-containing hydrophobic di-peptide motif, full-length type I IP_3 receptors do not interact with either FKBP12 or FKBP12.6 biochemically, suggesting that FKBP binding involves a combination of both the presence of the di-peptide motif and the proper local secondary structure of the interacting peptidylprolyl residues (19). An analogous proline-containing di-peptide motif is also founding RyR1, which has long been known to robustly bind FKBP12 (6, 7). More recently, it was reported that FKBP12 binding to RyR1 is abolished by substitution of a glycine, glutamate, or isoleucine residue for the valine residue at amino acid 2461 (Val-2461), which immediately precedes the conserved proline residue in this motif. In addition, mutations of Val-2461 that abolish FKBP12 binding to RyR1 (V2461G, V2461E, and V2461I) result in abnormal gating of mutant release channels monitored following incorporation into planar lipid bilayers (20). Interestingly, although mutation of V2641 to an isoleucine residue (which is the same amino acid found at the corresponding position of the cardiac RyR or RyR2) eliminates FKBP12 binding it permits binding to FKBP12.6, the cardiac FKBP isoform. Moreover, binding of FKBP12.6 to V2461I-containing release channels fully restores normal gating of the channel (20). Although FKBP binding to RyRs is strongly dependent upon this hydrophobic peptidy-prolyl sequence, this region could either directly participate in FKBP binding or represent an important modulator of the affinity of a site located in a different region of the protein. Gaburjakova *et al.* (20) suggested that FKBP12 might bind to and stabilize a twisted amide transition state intermediate of a peptidyl-prolyl bond formed by the Val-2461 and Pro-2462 residues of RyR1. Alternatively, reports from other groups have suggested that the primary site for FKBP binding maps to an entirely different region of RyR1 (21, 22).

Given potential problems related to nonspecific actions of rapamycin and FK506 (16, 23), we have used a molecular approach to specifically probe the influence of FKBP binding to RyR1 on EC coupling and bi-directional signaling between the DHPR and RyR1 in skeletal muscle. Accordingly, we have characterized orthograde and retrograde coupling in dyspedic myotubes following expression of mutant RyR1 proteins known to disrupt FKBP12 binding to RyR1 (*e.g.* V2461G and V2461I). Our results indicate that disruption of FKBP binding to RyR1 severely compromises voltage-gated SR Ca^{2+} release (orthograde coupling) without altering resting [Ca^{2+}], SR Ca^{2+} release triggered by maximal concentrations of caffeine or CPA, or RyR1-mediated enhancement of L-channel activity (retrograde coupling). Co-expression of FKBP12.6 and V2461I, which binds FKBP12.6 but not FKBP12, completely restored normal voltage-gated Ca^{2+} release in myotubes confirming that the reduction in voltage-gated Ca^{2+} release arises from alterations in the ability of RyR1 to bind FKBPs. These data indicate that FKBP12 binding to RyR1 enhances the gain of skeletal muscle EC coupling, possibly by stabilizing the open/closed states of single release channels and/or by ensuring efficient activation of non-DHPR-coupled SR Ca^{2+} release channels.

EXPERIMENTAL PROCEDURES

Preparation and cDNA Microinjection of Dyspedic Myotubes—Myotubes were prepared from primary culture of dyspedic muscle as described previously (24, 25). 4–7 days after the initial plating of myoblasts, nuclei of individual dyspedic myotubes were microinjected (24, 26) with cDNAs encoding CD8 (0.2 μg/μl) and either wild-type RyR1, V2461G, P2462Q, V2461G/P2462Q, or V2461I (0.5 μg/μl). In some experiments investigating effects of the V2461I mutation (see Fig. 4), 0.2 μg/μl of FKBP12.6 cDNA was also included in the microinjection solution. Expressing myotubes were subsequently identified 48–72 h postinjection by incubation with CD8 antibody-coated beads (Dynabeads, Dynal ASA, Oslo, Norway). Point mutations in RyR1 were constructed using a standard two-step site-directed mutagenesis strategy with the entire PCR-modified cDNA portion ultimately confirmed by sequence analysis.

Co-immunoprecipitation of FKBP12 and RyR1 Constructs—Dyspedic myotubes derived from the 1B5 dyspedic cell line (27) were infected with HSV-1 amplicon virions encoding RyR1, V2461G, V2461I or P2462Q at a multiplicity of infection (MOI) of 0.5 (28). Co-immunoprecipitation of endogenous FKBP12 and the expressed RyR1 construct was carried out 2 days after viral infection (27). Briefly, microsomes were isolated from infected myotubes and diluted with lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 12.5 μg/ml leupeptin, and 20 μg/ml pepstatin A). For control experiments, junctional SR (JSR) was isolated from rabbit skeletal muscle using standard techniques (29). The lysates were centrifuged, and protein concentration determined. Control Western blot experiments confirmed FKBP12 and RyR1 expression levels were similar following virion infection with each of the different RyR1 constructs (data not shown). 300 μg of total protein of the lysates was incubated overnight at 4 °C with 30.0 μl of anti-RyR monoclonal antibody (34C, Sutko and Airey, Developmental Studies Hybridoma Bank, The University of Iowa), followed by further incubation with protein G-Sepharose beads (Amersham Biosciences). Beads were then washed three times with lysis buffer to remove nonspecifically bound proteins. Immune complexes were treated with SDS-sample buffer (5% glycerol, 100 mM dithiothreitol, 2% SDS, 0.01% bromphenol blue, and 125 mM Tris, pH 6.8), split in half, and each half separately subjected to SDS-polyacrylamide electrophoresis (SDS-PAGE, 17% for FKBP12 and 6% for RyR1). Following electrophoresis, proteins from both gels were transferred to polyvinylidene difluoride membranes and blocked for 2 h at room temperature in 10% nonfat dry milk in TBST (TBS with 0.05% Tween 20), incubated for 1 h at room temperature with either an anti-FKBP12 polyclonal antibody (1:1000, Affinity Bioreagent Inc) or a anti-RyR monoclonal antibody (1:100). After washing, membranes were incubated with peroxidase-conjugated donkey anti-rabbit or goat anti-mouse IgG antibody (1:5000, Amersham Biosciences) for 60 min at room temperature, washed three times with TBST, and developed using enhanced chemiluminescence (SuperSigna®, Pierce).

Simultaneous Measurements of L-currents and Intracellular Ca^{2+} Transients—The whole-cell patch clamp technique was used to simultaneously measure L-currents and Ca^{2+} transients in myotubes in which RyR1 constructs were expressed via nuclear microinjection as described above (24–26). The speed and uniformity of the voltage clamp was maximized in these experiments by selecting morphologically compact myotubes containing 4–6 nuclei such that the time constant for charging the membrane capacitance ($\tau_m = R_S \times C_m$) was less than 0.5 ms and the maximal voltage error due to series resistance ($V_e = R_S \times I_{Ca}$) was always less than 5 mV. Inward L-currents were elicited by 30-ms test pulses of variable amplitude from a holding potential (HP) of −80 mV following a prepulse protocol used to inactivate T-type Ca^{2+} channels (1 s to −20 mV followed by 25 ms to −50 mV before each test pulse). L-currents were normalized to cell capacitance (pA/pF), plotted as a function of the membrane potential, and fitted according to Equation 1.

$$I = G_{max} \times (V_m - V_{rev})/(1 + \exp[(V_{G1/2} - V_m)/k_G]) \qquad \text{(Eq. 1)}$$

Relative changes in intracellular Ca^{2+} in patch clamp experiments were measured using an internal solution containing 0.2 mM Fluo-3, which does not significantly alter L-channel properties in cultured myotubes (24–26). After rupture of the cell membrane and entry into the whole-cell mode, a waiting period of 5 min was used to allow the dye to diffuse into the cell interior. A 75-watt xenon bulb and high speed DeltaRAM illuminator (Photon Technology Incorporated, Monmouth Junction, NJ) were used to excite the dye (480 ± 20 nm) present in a small rectangular region of the voltage-clamped myotube. A computer-controlled shutter was used to eliminate illumination during intervals between test pulses. Fluorescence emission was measured using a dichroic long-pass mirror centered at 505 nm, an emission filter centered at 535 ± 25 nm, and a photomultiplier detection system operating in analogue mode (Photon Technology Incorporated, Monmouth Junction, NJ). The background fluorescence was measured and cancelled by analog subtraction. Fluorescence traces were analog-filtered (τ = 0.5 ms) before digitization at 10 kHz and expressed as $\Delta F/F$, where F represents the baseline fluorescence immediately prior to depolarization, and ΔF represents the fluorescence change from baseline. The magnitude of maximal voltage-gated calcium release ($(\Delta F/F)_{max}$) was estimated by fitting the data according to Equation 2.

$$\Delta F/F = (\Delta F/F)_{max}/(1 + \exp[(V_{F1/2} - V_m)/k_F]) \quad \text{(Eq. 2)}$$

L-currents and intracellular Ca^{2+} transients were recorded using a pipette solution containing (in mM): 145 Cs-Aspartate, 10 CsCl, 0.1 Cs_2-EGTA, 1.2 $MgCl_2$, 5 Mg-ATP, 0.2 K_5-Fluo-3, 10 mM HEPES, pH 7.4. The external solution contained (in mM): 145 TEA-Cl, 10 $CaCl_2$, 0.003 TTX, and 10 HEPES, pH 7.4.

Measurements of Resting Ca^{2+} and Caffeine-induced Ca^{2+} Transients in Intact Myotubes—Intracellular Ca^{2+} was measured in intact (non-patched) myotubes loaded with the fluorescent Ca^{2+} indicator Indo-1 AM (Molecular Probes, Eugene, OR) as described previously (24, 26). Briefly, myotubes grown on glass coverslips were loaded by incubation with 6 μM indo-1 AM for 1 h at room temperature in a normal rodent Ringer solution (containing 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4). After removal of extracellular Indo-1 AM by washing the dish several times with Indo-free Ringer, myotubes were incubated for 20 min at 37 °C to allow for de-esterification of the dye. Coverslips of indo-1-loaded myotubes were mounted on an inverted Olympus IX70 microscope, bathed in Ringer solution, and excited at 350 ± 10 nm using a DeltaRam illumination system (Photon Technology Inc., Princeton, NJ). Fluorescence emission at 405 and 485 nm was monitored using a ×40 (1.35 NA) oil immersion objective, collected at 100 Hz using a photomultiplier detection system, and presented as the ratio (R) of F_{405}/F_{485}. Although this sampling rate (10 ms/pt) may somewhat underestimate the maximal value of rapid action potential-induced Ca^{2+} transients in intact myotubes, it was clearly sufficient to resolve the complete magnitude/time course of caffeine- and CPA-induced Ca^{2+} transients, as well as the declining phase of the rapid Ca^{2+} transients. The resting F_{405}/F_{485} ratio was converted to cytosolic Ca^{2+} levels using an *in situ* calibration approach described previously (24). Relative changes in global Ca^{2+} levels were subsequently monitored following application of maximal concentrations of either caffeine (10 mM) or cyclopiazonic acid (CPA, 30 μM) using a rapid perfusion system (Warner Instruments, Inc., Hamlin, CT) that permits fast, local application of agonist as well as rapid washout with control solution (24, 26). Because of previously reported threshold (or all-or-none) effects of caffeine in myotubes (30), myotubes were perfused with a concentration of caffeine (10 mM) that always produced a maximal caffeine response. Caffeine- and CPA-induced Ca^{2+} transients were not converted to free Ca^{2+} since Ca^{2+} levels above ~150 nM resulted in contractions that introduce movement artifacts into the *in situ* calibration (24). Multiple myotubes were measured per dish and bulk gravity perfusion (~10 ml) with control Ringer's was used to wash the dish between each measurement. Peak intracellular Ca^{2+} changes in response to agonist application are expressed as ΔRatio ($R_{agonist} - R_{baseline}$). Data were analyzed using FeliX (Photon Technology Inc., Princeton, NJ) and SigmaPlot 2000 (SPSS, Inc., Chicago, IL) software packages. Data are presented as mean ± S.E.

There are several reasons for the use of fluo-3 to monitor relative changes in myoplasmic Ca^{2+} during whole cell patch clamp experiments and indo-1 to monitor resting and drug-induced changes in intracellular Ca^{2+} in intact myotubes. Fluo-3 free acid, introduced via intracellular dialysis of whole-cell patch clamp myotubes, was used to monitor voltage-gated Ca^{2+} release because fluo-3: 1) exhibits a lower affinity for Ca^{2+} in intact cells than Indo-1 or Fura-2 (31) and therefore is less likely to be exhibit saturation at strong depolarizations, 2) is used by several other laboratories conducting similar research in the field and therefore allows for a more meaningful quantitative comparison of our data with that published previously (and in the future), and 3) is excited by wavelengths in the visible spectrum (*e.g.* 480 nm), and therefore can be conveniently used to monitor relative changes in Ca^{2+} in myotubes grown on plastic tissue culture dishes. On the other hand, Indo-1 was used to monitor resting Ca^{2+} and drug-induced elevations in resting Ca^{2+} in intact myotubes because Indo-1 is a ratiometric Ca^{2+} dye that exhibits a higher affinity for Ca^{2+} in intact cells and is therefore better suited for monitoring low resting Ca^{2+} levels (<100 nM) in myotubes (24). In addition, while several drugs (*e.g.* caffeine) significantly quench the fluorescence of single wavelength dyes such as Fluo-3, both wavelengths of the dual-emission dye Indo-1 are quenched to comparable levels (32) by caffeine, thus eliminating quench-induced artifacts in the resulting ratio.

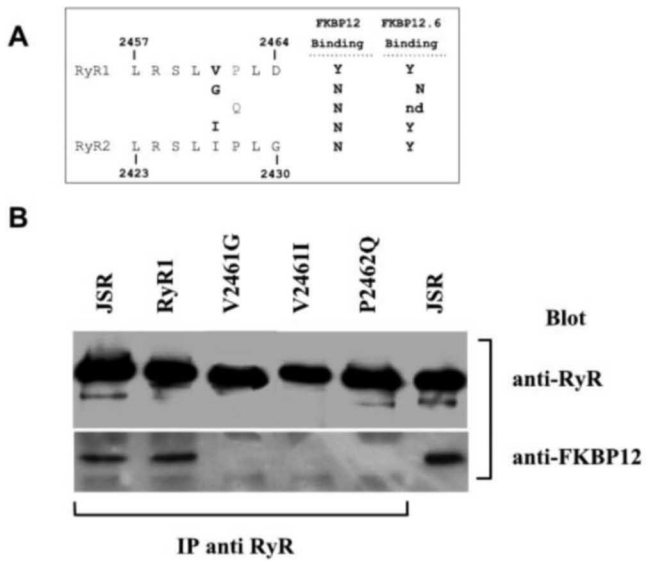

FIG. 1. **FKBP12 binding to RyR1 constructs.** *A*, amino acid substitutions at Val-2461 (*bold*) and Pro-2462 (*gray*) in RyR1 disrupt FKBP12 and FKBP12.6 (20) binding. The scheme is based on that shown in Refs. 18 and 20. *nd*, not determined. *B*, co-immunoprecipitations of FKBP12 with RyR1, V2461G, V2461I, and P2462Q were performed as described under "Experimental Procedures." Although the amount of expressed RyR1 was the same in each case (*upper lanes*), FKBP12 co-immunoprecipitated only with wild-type RyR1 but not with either V2461G, V2461I, or P2462Q (*lower lanes*). *JSR* lanes utilized 10 μg of purified junctional SR isolated from rabbit skeletal muscle (29).

RESULTS

Ryanodine receptors contain a highly conserved hydrophobic peptidyl-prolyl motif (L*X*PL; where *X* is either a V or I residue in RyR1 and RyR2, respectively) that strongly influences FKBP binding to these intracellular Ca^{2+} release channels (Fig. 1*A*) (20). The overall goal of our study was to assess the impact of FKBP binding to RyR1 on Ca^{2+} homeostasis, EC coupling, and DHPR-RyR1 bi-directional signaling in intact skeletal myotubes. Accordingly, we compared the properties of EC coupling in RyR1-null (dyspedic) myotubes following expression of either wild-type or FKBP binding-deficient RyR1 proteins. Because of controversy with regard to the identity of the precise FKBP binding site on RyR1 (see Introduction), we first set out to confirm that mutations to Val-2461 indeed disrupt FKBP binding following expression in cultured myotubes (Fig. 1*B*). For these experiments, dyspedic myotubes were infected with HSV-1 amplicon virions encoding RyR1, V2461G, V2461I, or P2462Q. FKBP12 expression in 1B5 myotubes was not affected by the expression of any of the RyR1 constructs (data not shown). Co-immunoprecipitation experiments were then performed on membrane fractions of transduced myotubes in order to determine if endogenous FKBP12 present in myotubes physically associates with each construct. Western blot analysis demonstrated that immunoprecipitation with an anti-RyR an-

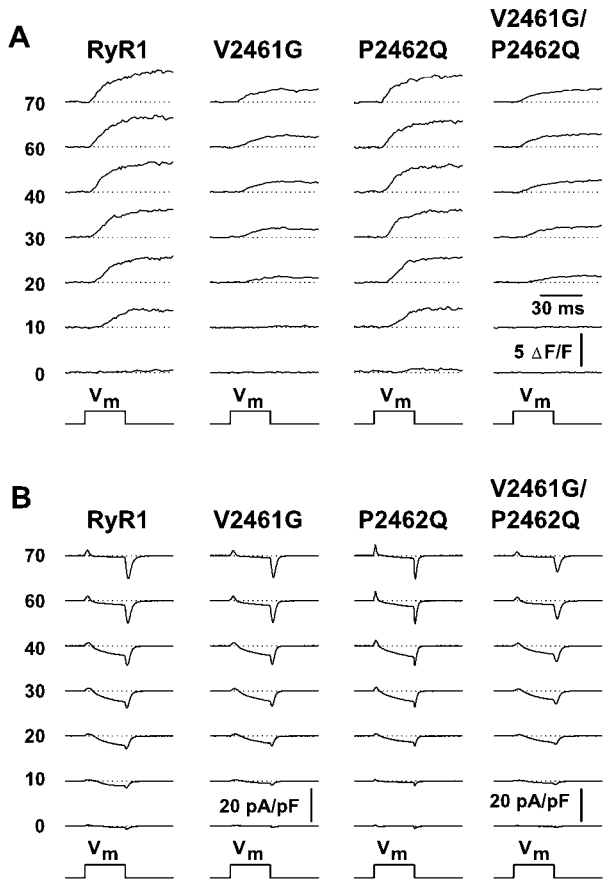

FIG. 2. **The V2461G mutation in RyR1 selectively reduces voltage-gated SR Ca^{2+} release.** Representative intracellular Ca^{2+} transients (*A*, $\Delta F/F$) and L-type Ca^{2+} currents (*B*, *pA/pF*) elicited by 30-ms test pulses to the indicated potentials (*left*) recorded from RyR1- (*column 1*), V2461G- (*column 2*), P2462Q- (*column 3*), and V2461G/P2462Q- (*column 4*) expressing dyspedic myotubes. Ca^{2+} transient and L-current traces at each voltage were obtained from the same myotubes for each construct.

tibody verified similar levels of expressed RyR1 in each of the membrane fractions (Fig. 1*B*, *upper lanes*). As expected, FKBP12 co-immunoprecipitated with wild-type RyR1 but not with either V2461G or V2461I (Fig 1*B*, *lower lanes*), thus confirming the results of Gaburjakova *et al.* (20). Interestingly, we also found that the P2462Q mutation also disrupted FKBP binding to RyR1 using this assay, a result analogous to that observed for the type 1 IP_3R (18).

To investigate the impact of FKBP binding to RyR1 on the orthograde and retrograde signals of skeletal muscle EC coupling, we used the whole-cell patch clamp technique in conjunction with the Ca^{2+} sensitive dye fluo-3 to simultaneously monitor L-type Ca^{2+} channel activity (DHPR function) and voltage-gated SR Ca^{2+} release (RyR1 function) in dyspedic myotubes in which RyR1 expression was achieved through nuclear microinjection of cDNAs encoding wild-type or FKBP binding-deficient RyR1 proteins (Fig. 2). Uninjected dyspedic myotubes exhibit very small L-currents and lack voltage-gated SR Ca^{2+} release (2, 3). Expression of wild-type RyR1 restored both voltage-gated SR Ca^{2+} release (orthograde coupling) and large L-type Ca^{2+} currents (retrograde coupling) (Fig. 2, *A* and *B*, *column 1*). Expression of V2461G FKBP binding-deficient RyR1 in dyspedic myotubes restored L-current density to a similar level as that attributable to wild-type RyR1 (Fig. 2*B*, *column 2* and Table I), indicating that V2461G release channels were synthesized, properly targeted to sarcolemmal-SR junctions, and functionally interacted with DHPRs present in these junctions. These results indicate that FKBP binding to RyR1 does not significantly influence retrograde signaling between RyR1 and the DHPR. However, maximal voltage-gated SR Ca^{2+} release was markedly reduced (~50%) and shifted to slightly more positive potentials (~6 mV) compared with that of wild-type RyR1 in these same V2461G-expressing myotubes (Fig. 2*A*, *column 2* and Table I).

Cameron *et al.* (18) demonstrated that specific mutations to a highly conserved proline residue in the hydrophobic peptidyl-prolyl motif of the type I IP_3R (*e.g.* P1401Q) results in the loss of FKBP binding and we found that endogenous FKBP12 fails to co-immunoprecipitate with a RyR1 mutant in which the analogous proline residue was mutated to a glutamine residue (P2462Q, Fig. 1*B*). Surprisingly, expression of P2462Q in dyspedic myotubes restored both voltage-gated SR Ca^{2+} release (orthograde coupling; Fig. 2*A*, *column 3*, and Table I) and L-type Ca^{2+} current density (retrograde coupling; Fig. 2*B*, *column 3* and Table I) to levels nearly identical to that of wild-type RyR1. However, similar to that found for V2461G, dyspedic myotubes expressing a double mutant that contained both the V2461G and P2462Q mutations (V2461G/P2462Q) exhibited maximal voltage-gated SR Ca^{2+} release that was markedly reduced (~50%) and slightly shifted to more positive potentials compared with that of wild-type RyR1 (Fig. 2*A*, *column 4* and Table I). In addition, expression of the V2461G/P2462Q double mutant fully restored L-channel function in a manner similar to that of wild-type RyR1, V2461G, and P2462Q (Fig. 2*B*, *column 4* and Table I). The effects of the V2461G and P2462Q mutations in RyR1 on the voltage dependence of voltage-gated SR Ca^{2+} release (Fig. 3, *A* and *B*) and L-type Ca^{2+} currents (Fig. 3*C*) are summarized in Fig. 3. These data demonstrate that the V2461G mutation, but not the P2462Q mutation, selectively reduced orthograde coupling between the DHPR to RyR1. In addition, the double mutant (V2461G/P2462Q) also caused an identical selective reduction in voltage-gated SR Ca^{2+} release, further confirming the critical role of Val-2461 in maximizing SR Ca^{2+} release during sarcolemmal depolarization.

Since the V2461G, but not the P2462Q, mutation reduced maximal voltage-gated SR Ca^{2+} release following expression in dyspedic myotubes, it is possible that the glycine mutation may cause a conformational alteration in RyR1 that alters release channel function in a manner that is independent of FKBP12 binding. Gaburjakova *et al.* (20) addressed this possibility by taking advantage of the fact that functional abnormalities associated with the V2461I mutation in RyR1 could be rescued by the addition of FKBP12.6. We have used a similar approach to determine the FKBP dependence of the reduction in orthograde coupling caused by mutations to V2461. First, we confirmed that orthograde coupling (Fig. 4, *A* and *B* and Table I), but not retrograde coupling (Fig. 4, *C* and *D* and Table I), is significantly reduced (~70%) in V2461I-expressing myotubes. Co-expression of FKBP12.6 did not alter voltage-gated SR Ca^{2+} release mediated by wild-type RyR1 or the augmentation of L-type Ca^{2+} current density produced by either RyR1 or V2461I (Fig. 4, *C* and *D* and Table I). However, co-expression of FKBP12.6 fully restored maximal voltage-gated SR Ca^{2+} release in V2461I-expressing myotubes (Fig. 4, *A* and *B* and Table I). The combination of ability of FKBP12.6 to both bind V2461I (20) and restore normal orthograde coupling mediated by V2461I (Figs. 4 and 5) provides compelling support for the conclusion that FKBP binding to RyR1 strongly influences voltage-gated SR Ca^{2+} during EC coupling in skeletal myotubes. The functional effects on maximal voltage-gated SR Ca^{2+} release of mutations within the conserved hydrophobic peptidyl-prolyl motif that disrupt FKBP binding to RyR1 are

TABLE I
Parameters of fitted I-V and ΔF/F-V curves

Values represent mean ± S.E. from the number of experiments indicated in column *n*.

	n	I-V data				ΔF/F-V data		
		G_{max}	$V_{G\ 1/2}$	k_G	V_{rev}	$(\Delta F/F)_{max}$	$V_{F\ 1/2}$	k_F
		nS/nF	*mV*	*mV*	*mV*		*mV*	*mV*
RyR1	31	146 ± 12	17.6 ± 0.9	5.8 ± 0.1	73 ± 1.5	3.0 ± 0.3	11.0 ± 1.2	5.4 ± 0.4
V2461G	29	155 ± 12	22.4 ± 1.0[a]	5.6 ± 0.1	79 ± 1.4	1.6 ± 0.2[a]	17.5 ± 1.1[a]	5.3 ± 0.4
P2462Q	16	157 ± 11	20.7 ± 1.2	5.8 ± 0.3	75 ± 2.0	3.1 ± 0.4	11.9 ± 1.8	5.0 ± 0.5
P2462Q + V2461G	14	191 ± 24	25.9 ± 1.1[a]	6.2 ± 0.2	82 ± 2.0[a]	1.5 ± 0.2[a]	19.4 ± 1.3[a]	5.1 ± 0.5
V2461I	8	122 ± 20	17.6 ± 1.9	6.0 ± 0.3	66 ± 3.0	0.9 ± 0.1[a]	13.4 ± 2.6	6.3 ± 0.6
V2461I/FKBP12.6	7	106 ± 10	19.2 ± 1.3	5.8 ± 0.2	74 ± 4.3	2.9 ± 0.6	13.1 ± 2.6	5.6 ± 0.5
RyR1/FKBP12.6	8	148 ± 18	18.7 ± 0.8	5.5 ± 0.2	76 ± 2.9	2.8 ± 0.5	11.9 ± 2.5	5.6 ± 0.9

[a] Compared to RyR1 $p < 0.005$.

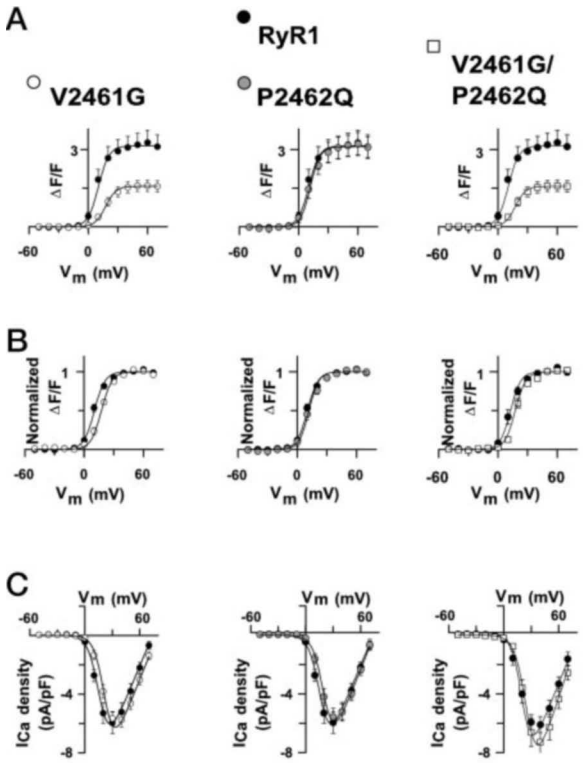

FIG. 3. **Voltage dependence of SR Ca^{2+} release and L-type Ca^{2+} currents.** *A*, average voltage dependence of peak intracellular Ca^{2+} transients for V2461G- (*left*, *open circles*), P2462Q- (*middle*, *gray circles*), and V2461G/P2462Q- (*right*, *open squares*) expressing dyspedic myotubes compared with that attributable to wild-type RyR1 (*closed circles*). The average values (±S.E.) for the parameters obtained by fitting each myotube within a group separately by Eq. 2 are given in Table I ($\Delta F/F$ − V data). The *solid lines* through the data were generated using Eq. 2, and the corresponding parameters given in Table I. *B*, the $\Delta F/F$ − V data and curves shown in *A* were normalized to their respective maximal values ($(\Delta F/F)_{max}$). *C*, average peak I-V curves for V2461I- (*left*, *open circles*), P2462Q- (*middle*, *gray circles*), and V2461I/P2462Q- (*right*, *open squares*) expressing dyspedic myotubes compared with that attributable to wild-type RyR1 (*closed circles*). The average values (±S.E.) for the parameters obtained by fitting each myotube within a group separately by Eq. 1 are given in Table I (I-V data). The *solid lines* through the data were generated using Eq. 1 and the corresponding parameters given in Table I.

summarized in Fig. 5. Fig. 5*A* highlights our observation that mutations to Val-2461, but not Pro-2462, which disrupt biochemical co-purification of FKBP12 with RyR1 (Fig. 1*B*) result in a marked reduction in maximal voltage-gated SR Ca^{2+} release. More importantly, coexpression of FKBP12.6 fully restored normal maximal Ca^{2+} release in V2461I-expressing myotubes, but not V2461G-expressing myotubes (Fig. 5*B*), consistent with the notion that FKBP binding to RyR1 potentiates voltage-gated SR Ca^{2+} release.

The observed reduction in voltage-gated SR Ca^{2+} release in V2461G- and V2461I-expressing myotubes could arise either from a decrease in the overall efficacy of orthograde coupling (*i.e.* ability of the voltage sensor to release SR Ca^{2+}) or a reduction in SR Ca^{2+} content that occurs secondary to increased SR Ca^{2+} leak through FKBP-deficient release channels (33, 34). In order to distinguish between these two possibilities we monitored resting Ca^{2+} levels and SR Ca^{2+} content in intact Indo-1 AM-loaded dyspedic myotubes expressing either wild-type RyR1, V2461G, or V2461I (Fig. 6). RyR1-, V2461G-, and V2461I-expressing dyspedic myotubes exhibited similar resting Ca^{2+} levels (Fig. 6*B*), which were higher than that of uninjected myotubes (24). Moreover, SR Ca^{2+} content was also similar in RyR1-, V2461G-, and V2461I-expressing myotubes as assessed from the increase in myoplasmic Ca^{2+} induced by exposure to maximal effective concentrations of either caffeine (10 mM) or cyclopiazonic acid (CPA, 30 μM), a reversible inhibitor of SR Ca^{2+}-ATPase pumps (Fig. 6, *A* and *B*). Finally, resting Ca^{2+} levels were not significantly different under any of these conditions even after caffeine removal (data not shown). The results in Fig. 6 indicate that disruption of FKBP binding to RyR1 does not result in a significant leak of SR Ca^{2+} in skeletal myotubes, indicating that marked SR Ca^{2+} depletion does not account for the reduction in voltage-gated SR Ca^{2+} release observed for mutations in Val-2461 that result in a disruption in FKBP12 binding to RyR1 (Figs. 2–4).

Two distinct types of spontaneous global Ca^{2+} transients were observed in intact indo-1 AM-loaded myotubes (see Fig. 6*A*). Occasionally (only 15/185 myotubes; see Fig. 6*A*, *top left*), expressing myotubes exhibited large, slowly activating Ca^{2+} transients (defined as taking >500 ms at half-maximal amplitude) that presumably reflect Ca^{2+}-induced Ca^{2+}-release (CICR). Similar slow CICR transients were usually observed during caffeine application (63/70 myotubes; see Fig. 6*A*, *bottom*). A significant percentage of expressing myotubes (27/185 myotubes) exhibited smaller, rapid global Ca^{+} transients characterized by a rapid rise (~20 ms) and decay (~200 ms) and generally of smaller amplitude than peak CICR or caffeine-induced Ca^{2+} transients (see rapid events in Fig. 6*A*). Thus, the rapid global Ca^{2+} release events likely arise from spontaneous electrical activity that result from DHPR activation of SR Ca^{2+} release channels. It is important to note that these rapid events represent global Ca^{2+} changes (as opposed to localized Ca^{2+} sparks) since they lasted too long to result from truly localized Ca^{2+} release events and were observed using indo-1 and conventional (non-confocal) fluorescence microscopy. Since dissociation of FKBP12 from RyR1 results in increased release channel sensitivity to Ca^{2+}-dependent activation (9), we evaluated the effects of the V2461G mutation on the properties of Ca^{2+} oscillations observed in both the absence and presence of caffeine. The frequency (21.7 ± 4.7 and 24.4 ± 9.1 min^{-1}) and duration (217 ± 22 and 205 ± 38 ms) of spontaneous rapid

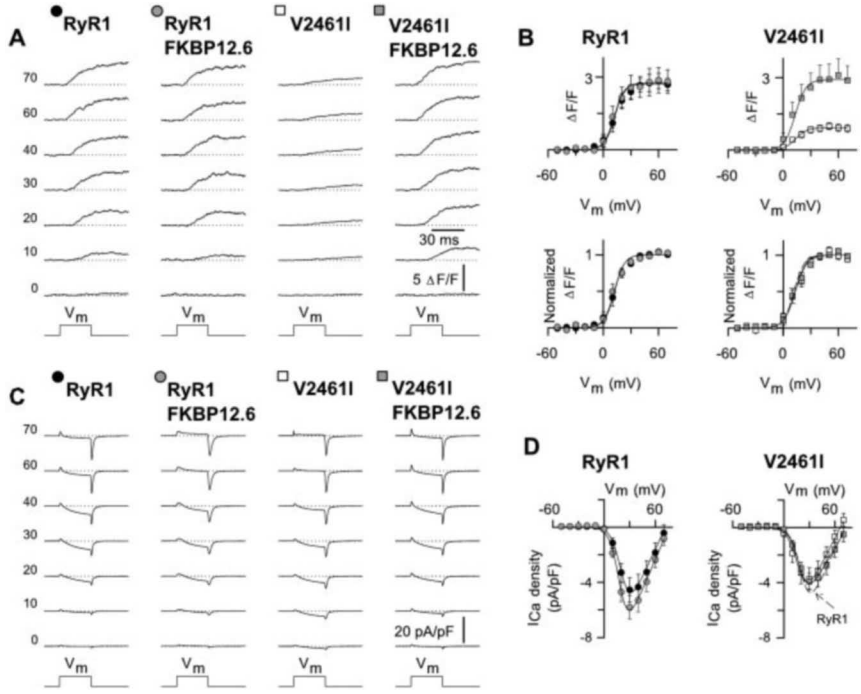

FIG. 4. **Co-expression of FKBP12.6 restores voltage-gated SR Ca^{2+} release in V2461I-expressing myotubes.** *A*, representative Ca^{2+} transients recorded from RyR1- (*closed circles*), RyR1 plus FKBP12.6- (*gray circles*), V2461I- (*open squares*), and V2461I plus FKBP12.6- (*gray squares*) expressing dyspedic myotubes. *B*, average peak (*upper*) and normalized (*lower*) voltage-gated Ca^{2+} release observed for dyspedic myotubes expressing RyR1 (*left*) or V2461I (*right*) in the absence or presence to FKBP12.6 (*gray symbols*). *C*, representative L-type Ca^{2+} currents recorded from RyR1- (*closed circles*), RyR1 plus FKBP12.6- (*gray circles*), V2461I- (*open squares*), and V2461I plus FKBP12.6- (*gray squares*) expressing dyspedic myotubes. For each construct, the illustrated traces are taken from the same myotubes as those shown in *A*. *D*, average voltage dependence of L-channel activation for the constructs shown in *C*.

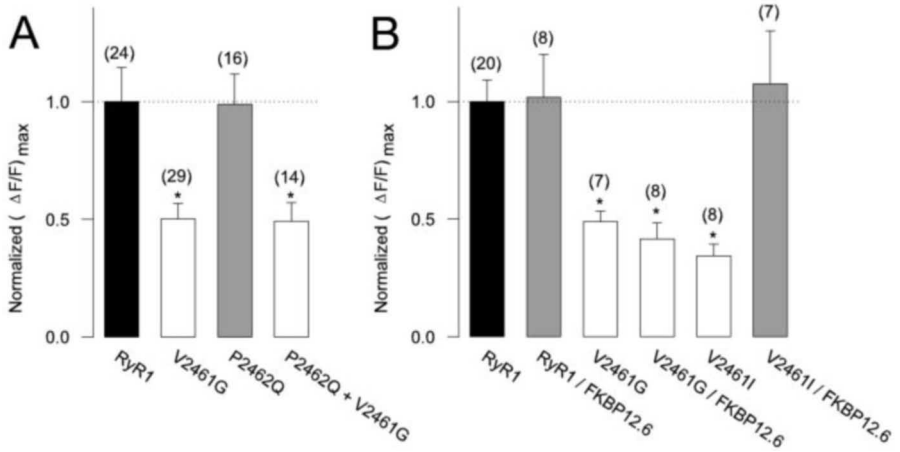

FIG. 5. **FKBP binding to RyR1 promotes voltage-gated Ca^{2+} release.** *A* and *B*, the maximal amplitude of voltage-gated Ca^{2+} release ($(\Delta F/F)_{max}$) for each construct was normalized to the respective culture-matched control values for RyR1 (*A*, 3.1 ± 0.5; *B*, 2.2 ± 0.2). *, $p < 0.05$.

global events were not different for RyR1- and V2461G-expressing myotubes, respectively. Interestingly, the spontaneous rapid global Ca^{2+} transients were of smaller amplitude in V2461G- (0.23 ± 0.04) compared with that of RyR1-expressing (0.40 ± 0.05) myotubes, consistent with results obtained from voltage clamp experiments. A similar analysis could not be quantified for V2461I since only 2/35 V2461I-expressing myotubes exhibited rapid spontaneous events. In addition, the vast majority of both RyR1- (29/33), V2461G- (22/22), and V2461I-expressing (12/15) myotubes exhibited slow CICR Ca^{2+} oscillations of similar frequency (9.9 ± 1.8, 10.6 ± 1.3, and 12.0 ± 2.9 min^{-1}, respectively) during the application of 10 mM caffeine (as seen in Fig. 6*A*). However, the slow caffeine-induced Ca^{2+} oscillations were significantly briefer (3.0 ± 0.4, 1.7 ± 0.2, and 1.5 ± 0.3 s for RyR1, V2461G, and V2461I, respectively) and larger in amplitude (0.27 ± 0.03, 0.40 ± 0.03, and 0.43 ± 0.6 s for RyR1, V2461G, and V2461I, respectively) in V2461G- and V2461I-expressing myotubes. These results indicate that FKBP binding to RyR1 influences the magnitude of both spontaneous rapid EC coupling-mediated Ca^{2+} transients and slow CICR-mediated Ca^{2+} oscillations observed during caffeine exposure.

DISCUSSION

Considerable evidence indicates that FKBP12 modulates RyR1 function. The majority of data implicating functional effects of FKBP12 on SR Ca^{2+} release channel activity have been obtained in artificial and/or non-cellular systems (8–10, 12). However, a few reports have evaluated the effects of disrupting the interaction between FKBP12 and RyR1 on EC coupling in "intact" skeletal muscle. Brillantes *et al.* (9) found that treatment of rat soleus muscle with FK506 or rapamycin-induced small amplitude twitches in the absence of stimuli and potentiated caffeine-induced contractures. Lamb and Stephenson (16) reported that treatment with FK506 or rapamycin caused a loss of depolarization-induced contractions that occurred in the absence of alterations in SR Ca^{2+} store content in mechanically skinned rat skeletal muscle fibers. These results support the notion that FKBP12 stabilizes the closed state of the release channel and that FKBP12 facilitates voltage sensor activation of SR Ca^{2+} release in intact skeletal muscle. However, conclusions derived from the studies of Brillantes *et al.* (9) and Lamb and Stephenson (16) relied on the use of high concentrations of the immunosuppressant drugs, FK-506 or rapamycin. Although treatment with FK506 or rapamycin has been

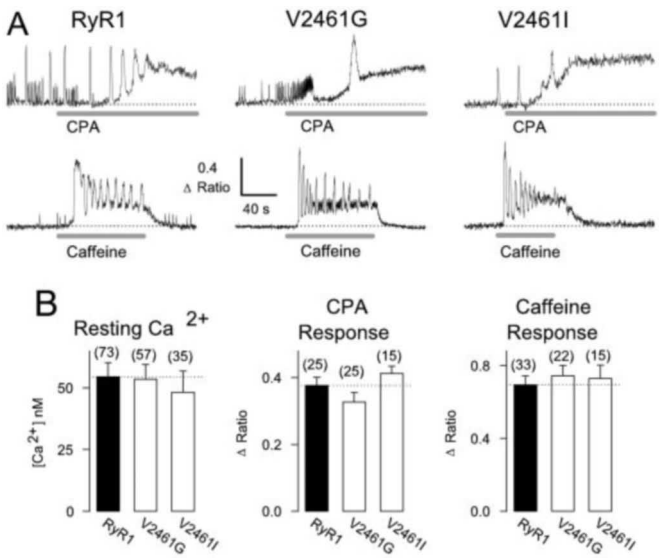

FIG. 6. **The V2461G mutation in RyR1 does not cause SR Ca^{2+} depletion.** *A*, representative CPA- (30 μM, *upper traces*) and caffeine- (10 mM, *lower traces*) induced Ca^{2+} responses in Indo-1 AM-loaded dyspedic myotubes expressing either RyR1 (*left*), V2461G (*middle*), or V2461I (*right*). The percentage of RyR1-, V2461G-, and V2461I-expressing myotubes that exhibited fast spontaneous Ca^{2+} oscillations was 25% (18 of 73), 12% (7 of 57), and 6% (2 of 35) respectively. In addition, spontaneous Ca^{2+} oscillations observed in V2461G-expressing myotubes were ~40% smaller (0.40 ± 0.05 versus 0.23 ± 0.04; $p < 0.05$) than those observed for wild-type RyR1. *B*, average values for resting Ca^{2+} (*left*), the steady-state CPA response (*middle*), and peak caffeine response (*right*) were not different for RyR1-, V2461G- (*middle*), and V2461I- (*right*) expressing dyspedic myotubes.

used as the "gold-standard" for extracting FKBPs from RyRs, effects of these drugs on EC coupling unrelated to this action have also been reported (16, 23). In fact, we have found that incubation of normal myotubes with either rapamycin or FK-506 (20 μM) significantly reduced voltage-gated Ca^{2+} release, as well as L-current magnitude and DHPR-associated gating currents[2] making it difficult to determine specific effects of the drugs on orthograde coupling.

In order to probe effects specifically related to FKBP binding to RyR1, we have used a drug-free molecular approach that involves functional characterization of effects on EC coupling following expression of FKBP12 binding-deficient mutant RyR1 proteins in dyspedic myotubes. Our results are the first to indicate that mutations to V2461 (V2461G and V2461I), which abolish FKBP binding to RyR1 lead to a large and selective reduction in voltage-activated SR Ca^{2+} release in a manner that and does not significantly alter resting Ca^{2+}, SR Ca^{2+} content, or the degree of retrograde coupling from RyR1 to the DHPR. We have previously shown that dyspedic myotubes that express RyR1 mutant proteins harboring certain central core disease (CCD) mutations (*e.g.* Y523S, R2163H, R2435H, and R164C) exhibit an increased SR Ca^{2+} leak that manifests as an elevation in resting myoplasmic Ca^{2+} concentration coupled with depleted SR Ca^{2+} stores (seen as a reduction in CPA and caffeine responsiveness) (26). However, the decrease in maximal voltage-gated SR Ca^{2+} release in myotubes expressing FKBP binding-deficient release channels does not appear to arise from a similar reduction in SR Ca^{2+} content since both V2461G- and V2461I-expressing myotubes exhibit normal resting Ca^{2+} levels and responses to caffeine and CPA that do not differ from those of wild-type RyR1-expressing myotubes. Thus, in contrast to increased SR Ca^{2+} leak proposed to occur following PKA-mediated hyperphosphorylation and subsequent dissociation of FKBP12 from for RyR1 (34) or FKBP12.6 from the cardiac RyR isoform (RyR2) (33), expression of FKBP12 binding-deficient RyR1 proteins in dyspedic myotubes does not lead to an increased SR Ca^{2+} leak and subsequent SR Ca^{2+} store depletion. Rather, the reduced ability of the DHPR to maximally activate Ca^{2+} release through FKBP binding-deficient channels indicates that a physiologic role of the FKBP12-RyR1 interaction is to regulate the gain of EC coupling in skeletal muscle.

How might FKBP12 enhance the gain of skeletal muscle EC coupling? There are at least three possible mechanisms. First, the binding of FKBPs to RyR1 could promote mechanical coupling from the DHPR to RyR1. Recent reports indicate that a peptide derived from the skeletal L-channel II-III loop (peptide A) directly activates RyR1 release channels and this activation is substantially reduced upon FKBP12 removal (14, 15). However, skeletal muscle EC coupling is unaltered following expression of DHPR constructs in dysgenic myotubes in which the peptide A sequence was either scrambled (35), deleted (36), or replaced by highly divergent II-III loop sequence obtained from the DHPR of the housefly, *Musca domestica* (37), results that seriously call into question the validity and physiologic relevance of the peptide A studies. An FKBP-mediated increase in the sensitivity of orthograde coupling also seems unlikely because the voltage dependence of SR Ca^{2+} release for the FKBP binding-deficient channels is shifted only modestly for V2461G and not at all for V2461I. In addition, any FKBP-mediated effects on DHPR-RyR1 coupling would need to be unidirectional since retrograde coupling is unaffected. Second, FKBP binding to RyR1 could serve to promote DHPR-activated Ca^{2+} release by ensuring that activated release channels open to their full conductance levels during depolarization. It has been suggested that in the absence of FKBP12, RyR1 channels monitored in planar lipid bilayers exhibit partial openings to levels referred to as subconductance states (8–10, 12). If FKBP12 deficient release channels in intact myotubes open primarily to subconductance levels upon activation by the DHPR, then voltage-gated SR Ca^{2+} release might be smaller and still exhibit a voltage dependence like that of wild-type RyR1, a result similar to that observed here. However, the absence of large effects on maximal caffeine-induced Ca^{2+} release for FKBP binding-deficient mutants (Fig. 6) and recent suggestions that the major effect of FKBP12 is to modulate RyR1 channel gating frequency (20) argue against altered subconductance activity as the primary mechanism for the decrease in the gain of EC coupling. Third, in addition to stabilizing the gating of individual release channels, FKBP12 also enhances the cooperative gating of neighboring release channels, resulting in the simultaneous or "coupled gating" of adjacent, physically connected release channels (11). Morphological evidence from freeze-fracture studies indicates that in junctional SR of skeletal muscle, only every other RyR is associated with a tetrad of DHPRs (38). Thus, FKBP-controlled coupled gating of adjacent release channels is one possible mechanism by which non-DHPR-coupled release channels could be activated during EC coupling (11). In this case, disruption of FKBP binding to RyR1 might result in junctions in which primarily DHPR-coupled release channels are activated during depolarization, thus reducing the gain of EC coupling.

A surprising observation in our experiments was that although FKBP12 did not co-immunoprecipitate with P2462Q (Fig. 1*B*), voltage-gated SR Ca^{+} release was normal for myotubes expressing this mutant (Figs. 2 and 3). The discordance between the ability of the P2462Q mutation to disrupt FKBP binding in biochemical experiments while not significantly altering orthograde coupling assessed in intact myotubes could

[2] G. Avila and R. T. Dirksen, unpublished observation.

be explained by in several ways. On the one hand, these results might suggest that FKBP binding to RyR1 does not influence EC coupling. However, the reduction in voltage-gated Ca^{2+} release in V2461I-expressing myotubes and complete restoration of voltage-gated release by coexpression of an FKBP isoform known to readily bind to this mutant provides a compelling argument against this assertion. Alternatively, the P2462Q mutation may only moderately reduce the affinity of RyR1 for FKBP12. The recent observation that the analogous mutation in RyR2 (P2428Q) fails to disrupt the binding of FKBP12.6 to RyR2 (22) supports the notion that the proline residue of the conserved hydrophobic peptidyl-prolyl motif is less important than the preceding residue (valine in RyR1 or isoleucine in RyR2) for the binding of FKBPs to RyRs. Thus, mutations that only marginally reduce the affinity of RyR1 for FKBP12 (*e.g.* P2462Q) may not disrupt FKBP binding in intact myotubes, but may be sufficient to prevent co-immunoprecipitation of the two proteins during biochemical purification procedures. Finally, although somewhat unlikely, it is also possible that the P2462Q mutation could impart two diametrically opposed actions: a reduction in orthograde coupling resulting from disruption in FKBP binding to RyR1 and a direct stimulatory effect on EC coupling of comparable magnitude. Experiments designed to directly assess the affinity of P2462Q for binding FKBP12 will be required to differentiate between these possibilities. In addition, rescue of FKBP12.6 binding and restoration (or possible enhancement) of voltage-gated Ca^{2+} release in myotubes expressing a V2461I/P2462Q double mutant could be used to probe for possible FKBP-independent stimulatory effects of the P2462Q mutation. Thus, additional lines of experimentation will be required in order to provide a more conclusive mechanistic explanation for why the P2462Q mutation disrupts FKBP binding in IP experiments but is without effect on EC coupling assessed in intact cells.

Potassium depolarization of dyspedic myotubes has previously been shown to evoke Ca^{2+} waves that are mediated by Ca^{2+} release through IP_3 receptors (39). However, these IP_3 receptor-mediated Ca^{2+} elevations are extremely slow (taking ~10 s to peak) and prolonged in duration (lasting up to 30–60 s). Moreover these slow Ca oscillations are limited primarily to perinuclear regions and do not readily propagate through adjacent cytoplasmic regions of the cell. The slow, long lasting, and localized nature of these IP_3 receptor-mediated Ca^{2+} oscillations make them highly unlikely to contribute to the rapid global EC coupling Ca^{2+} release events elicited by the brief 30-ms voltage clamp depolarizations used in our study (as shown in Figs. 2–5) or even during relatively rapid caffeine-induced Ca^{2+} oscillations (~2–4 s in duration) observed in intact Indo-1 AM-loaded myotubes (Fig. 6). Slow IP_3 receptor-mediated nuclear Ca^{2+} oscillations also probably contribute negligibly to prolonged elevations in Ca^{2+} induced by CPA because: 1) the fluorescence recording window in our experiments is set to monitor Ca^{2+} changes through the majority (*i.e.* nuclear and non-nuclear regions) of the myotube, 2) unlike potassium depolarization, CPA (or caffeine) is not thought to increase IP_3 levels in myotubes, and 3) CPA-induced Ca^{2+} increases persist much longer (>10 min) than a typical IP_3 receptor-mediated nuclear Ca^{2+} oscillation (~30 s). Nevertheless, our experiments cannot completely rule out a minor contribution of slowly increasing elevations in nuclear Ca^{2+} to the prolonged CPA-induced elevations in cytoplasmic Ca^{2+}.

The current work represents the first use of a targeted molecular approach to selectively disrupt FKBP12 binding to RyR1 to probe the influence of this interaction on skeletal muscle Ca^{2+} homeostasis and EC coupling. Our findings provide several critical pieces of new information in this regard: 1) mutations to Val-2461 disrupt FKBP12 binding to RyR1 following expression in cultured myotubes (a result similar to that reported for HEK293 cells, Ref. 20), 2) FKBP12 binding to RyR1 maximizes voltage-gated SR Ca^{2+} release during EC coupling (orthograde coupling), 3) the FKBP12-RyR1 interaction does not influence RYR1-mediated enhancement of L-type Ca^{2+} channel activity (retrograde coupling), and 4) despite the increased Ca^{2+} sensitivity, open probability, and gating frequency of FKBP binding-deficient RyR1 channels monitored in planar lipid bilayers (9, 20, 33), resting Ca^{2+} and SR Ca^{2+} content (reflected in maximal caffeine/CPA responses) is surprisingly similar for dyspedic myotubes expressing wild-type and FKBP binding-deficient release channels. These data indicate that FKBP12 is an important regulator of the gain of skeletal muscle EC coupling since molecular disruption of the FKBP12-RyR1 interaction in skeletal myotubes markedly reduces voltage-gated Ca^{2+} release in a manner independent of a quantifiable change in SR Ca^{2+} content. Clearly, it will be important for future work to determine the degree to which the decreased gain of skeletal muscle EC coupling following disruption of the FKBP12-RyR1 interaction involves alterations in the fundamental properties (*e.g.* magnitude, frequency, duration) of Ca^{2+} sparks observed in skeletal myotubes.

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