Kinetics of FKBP12.6 Binding to Ryanodine Receptors in Permeabilized Cardiac Myocytes and Effects on Ca Sparks

Tao Guo,* Razvan L. Cornea,* Sabine Huke, Emmanuel Camors, Yi Yang, Eckard Picht, Bradley R. Fruen, Donald M. Bers

Rationale: FK506-binding proteins FKBP12.6 and FKBP12 are associated with cardiac ryanodine receptors (RyR2), and cAMP-dependent protein kinase A (PKA)-dependent phosphorylation of RyR2 was proposed to interrupt FKBP12.6-RyR2 association and activate RyR2. However, the function of FKBP12.6/12 and role of PKA phosphorylation in cardiac myocytes are controversial.

Objective: To directly measure in situ binding of FKBP12.6/12 to RyR2 in ventricular myocytes, with simultaneous Ca sparks measurements as a RyR2 functional index.

Methods and Results: We used permeabilized rat and mouse ventricular myocytes, and fluorescently-labeled FKBP12.6/12. Both FKBP12.6 and FKBP12 concentrate at Z-lines, consistent with RyR2 and Ca spark initiation sites. However, only FKBP12.6 inhibits resting RyR2 activity. Assessment of fluorescent FKBP binding in myocyte revealed a high FKBP12.6-RyR2 affinity ($K_a=0.7\pm0.1\ \text{nmol/L}$) and much lower FKBP12-RyR2 affinity ($K_a=206\pm70\ \text{nmol/L}$). Fluorescence recovery after photobleach confirmed this $K_a$ difference and showed that it is mediated by $k_{off}$. RyR2 phosphorylation by PKA did not alter binding kinetics or affinity of FKBP12.6/12 for RyR2. Using quantitative immunoblots, we determined endogenous [FKBP12] in intact myocytes is $\approx 1\ \mu\text{mol/L}$ (similar to [RyR]), whereas [FKBP12.6] is $\leq 150\ \text{nmol/L}$.

Conclusions: Only 10% to 20% of endogenous myocyte RyR2s have FKBP12.6 associated, but virtually all myocyte FKBP12.6 is RyR2-bound (because of very high affinity). FKBP12.6 but not FKBP12 inhibits basal RyR2 activity. PKA-dependent RyR2 phosphorylation has no significant effect on binding of either FKBP12 or 12.6 to RyR2 in myocytes. (Circ Res. 2010;106:1743-1752.)

Key Words: FKBP12.6 ■ FKBP12 ■ Ca sparks ■ binding properties ■ rapamycin ■ RyR2

Cardiac ryanodine receptors (RyR2) are sarcoplasmic reticulum (SR) Ca release channels, crucial in excitation–contraction coupling. Dysfunctional RyR2, exhibiting enhanced Ca leak has been implicated in arrhythmogenesis and heart failure (HF). Homotetrameric RyR2s have a transmembrane channel domain, and regulatory and scaffold- cytoplasmic domain.

In heart, FK506 binding protein (FKBP) isoforms FKBP12 and FKBP12.6 are coexpressed and can bind RyR2 at a stoichiometry of 4 FKBP per RyR tetramer. FKBP12 binds RyR2 with much lower affinity, but is much higher in concentration in heart than FKBP12.6. Nevertheless, FKBP12 and 12.6 share 85% sequence homology and similar 3D structures. Human and rat FKBP12 differ in only 3 residues, and human and rat FKBP12.6 are identical. This makes study of human FKBP function in rat myocytes reasonable.

Effects of FKBP12s on RyR2 activity in myocytes are controversial. Some groups reported that dissociation of FKBP12.6 from RyR2 by immunosuppressants (rapamycin or FK506) activated RyR2 channels and induced subconductance states. RyR2 point mutations associated with cardiac sudden death may also exhibit altered FKBP12.6 interaction, and FK506 can alter resting Ca$^{2+}$ spark frequency (CaSpF) and SR Ca$^{2+}$ content. FKBP12.6 overexpression increased SR load and enhanced contraction. However, others reported that FKBP12.6 removal had no effect on RyR2 activity and failed to observe RyR2 subconductance states in channels from FKBP12.6 knockout mice. Some groups suggest that FKBP12 affects RyR2 differently from FKBP12.6. Therefore, FKBP12/12.6 binding and RyR2 effects remain unclear, especially in the cardiomyocyte environment (as studied here).

Altered FKBP-RyR2 interaction is a prominent hypothesis explaining increased SR Ca leak in HF via RyR2 hyperphos-
phorylation and FKBP dissociation from RyR2. However, Li et al found that cAMP-dependent protein kinase A (PKA)-dependent RyR2 phosphorylation did not alter resting RyR2 function in mouse myocytes without phospholamban (PLB) and increased SR Ca load. Xiao et al reported that PKA function in mouse myocytes without phospholamban (PLB) dependent RyR2 phosphorylation did not alter resting RyR2 activity; and endogenous FKBP was not functional in the absence of PLB.20 Li et al found that cAMP-dependent protein kinase A (PKA)-dependent phosphorylation of RyR2; and (4) endogenous FKBP12.6/KO in mouse myocytes without phospholamban (PLB) dependent RyR2 phosphorylation did not alter resting RyR2 activity; and endogenous FKBP12.6/KO in mouse myocytes without phospholamban (PLB) dependent RyR2 phosphorylation did not alter resting RyR2 activity.

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>binding maximum</td>
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<tr>
<td>CaSpF</td>
<td>calcium spark frequency</td>
</tr>
<tr>
<td>Di-8-ANEPPS</td>
<td>di-8-ANEPPS</td>
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<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
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<tr>
<td>FKBP12.6-KO</td>
<td>FKBP12.6 knockout</td>
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<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
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<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
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<tr>
<td>HF</td>
<td>heart failure</td>
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<tr>
<td>k&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>rate constant</td>
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<tr>
<td>OA</td>
<td>okadaic acid</td>
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<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase A</td>
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<tr>
<td>PLB</td>
<td>phospholamban</td>
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<td>PLB-KO</td>
<td>phospholamban knockout</td>
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<tr>
<td>Rhod-2</td>
<td>rhod-2</td>
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<tr>
<td>ROI</td>
<td>region of interest</td>
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<tr>
<td>RyR2</td>
<td>cardiac ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic/endoplasmic reticulum calcium ATPase</td>
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<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>τ</td>
<td>time constant</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>WT-FKBP</td>
<td>nonfluorescent wild-type FKBP</td>
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**Methods**

Rat and mouse ventricular myocytes were isolated and permeabilized as previously described (see the Online Data Supplement, available at http://circres.ahajournals.org).23 F-FKBP12.6 and F-FKBP12 were characterized using circular dichroism spectroscopy and ligand-binding studies (Online Figure I).24 Consistent with previous reports,23 F-FKBP constructs have the same secondary structure, RyR binding affinity and RyR1 effects as unlabeled and wild-type (WT) FKBP.

**Results**

**Localization of FKBP12.6/12 in Permeabilized Ventricular Myocytes**

Saponin-permeabilized rat ventricular myocytes were incubated with F-FKBP12.6 or 12. Figure 1A shows a confocal myocyte image with 20 nmol/L F-FKBP12.6 (note that bath supply of F-FKBP12.6 is inexhaustible). The cross-striated sarcomeric pattern colocalizes with Di-8-ANEPPS (Online Figure III), used to identify transverse tubules at the Z-line. Figure 1B shows that line scan fluorescence intensity peaks at Z-lines exhibit an interpeak distance (∼2 μm) that matches sarcomere length. The same striation pattern was seen for F-FKBP12 (Online Figure II), but with lower contrast, because of lower RyR2 affinity (ie, bath F-FKBP12 fluorescence is higher when RyR2 saturates). Preincubation with WT-FKBP12.6 (100 nmol/L) completely prevented the appearance of striations, indicating that F-FKBP12.6/12 bind to the same sites as WT-FKBP12.6, and that there is little nonspecific binding. Ca sparks can be measured simultaneously using rhod-2, and superimposition of F-FKBP and spark images (Figure 1C and 1D) shows that Ca spark initiation sites coincide with F-FKBP12.6 localization (Figure 1E). These data indicates that F-FKBP12.6/12 bind at the Z-line region where SR Ca release initiates during Ca sparks.

**Effects of cAMP and Rapamycin on FKBP Binding and Resting SR Ca Release**

Ca sparks are fundamental SR release events during rest and excitation–contraction coupling.1 and are a readout of elementary RyR2 function in myocytes. We simultaneously measured Ca sparks and F-FKBP12.6/12 association and dissociation, providing direct online correlation between FKBP-RyR2 interaction and functional consequences.

Figure 2A shows FKBP12 (1 μmol/L) wash-in and Z-line binding (expressed as the difference between Z-line and M-line fluorescence). F-FKBP12 wash-in was rapid, reaching apparent steady state in 2 to 3 minutes. Rapamycin (20 μmol/L) caused rapid F-FKBP12 wash-out (rapid decrease of Z-line fluorescence) despite a constant bath [F-FKBP12], but was similar in kinetics to simple wash-out of F-FKBP12. Neither F-FKBP12 nor rapamycin significantly altered CaSpF (Figure 2B), although rapamycin increased spark duration (Figure 2F). Small apparent decreases in spark amplitude and width were seen with both FKBP12 and rapamycin (Figure 2D and 2E). These might reflect real FKBP12 actions on RyR2, but the fact that both addition (+FKBP12) and removal (+rapamycin) had identical effects, makes us think these (and any tendency of CaSpF change in FKBP12) are not simply attributable to FKBP12. Moreover, wash-out of bath F-FKBP12 caused dissociation of F-FKBP12 from the Z-line (Figure 2A) without any change in CaSpF (not...
shown). This suggests that dissociation of FKBP12 from RyR2 had little effect on RyR2 activity (consistent with previous reports\textsuperscript{15,16}). Parallel studies using nonfluorescent WT FKBP12 also showed no effect on CaSpF (109±7% of control; rapamycin 105±8% of control), and both FKBP12 and rapamycin caused similar slight decreases in Ca spark amplitude and width (12 and 6% respectively; \( P<0.05 \)), whereas spark duration was unaltered (96% to 97% of control).

Activation of PKA by cAMP to phosphorylate RyR2 (as in\textsuperscript{20}) did not change FKBP12 binding (Figure 2A), but increased CaSpF by >50%. We previously showed that this CaSpF enhancement was entirely attributed to PLB phosphorylation and consequently increased SR Ca load.\textsuperscript{20} Therefore, these data suggest that cAMP-dependent PKA phosphorylation does not alter FKBP12 binding to RyR2. Note that bath F-FKBP is an inexhaustible pool, and that RyR2 saturation in intact cells may differ.

Figure 3A shows F-FKBP12.6 (100 nmol/L) wash-in, and Z-line binding also rapidly reached steady state (≈2 minutes). F-FKBP12.6 binding to RyR2 was paralleled by a small, but highly significant decrease in CaSpF (18%) and a logically consequent increase in SR Ca load (of similar percent, Figure 3B). Spark amplitude and duration were also decreased (Figure 3D and 3F). These data agree with reports that...
FKBP12.6 inhibits resting RyR2, thus enhancing SR Ca content. Using WT-FKBP12.6 (unlabeled), we found similar results (24±5% decrease in CaSpF, 15±1% decrease in spark amplitude; unaltered width and duration). Addition of cAMP/okadaic acid (OA) did not significantly alter F-FKBP12.6 binding (Figure 3A), but increased both CaSpF and SR Ca load (Figure 3B). Because F-FKBP12.6 binding was unaltered by cAMP, its dissociation cannot explain the enhanced CaSpF. Moreover, a primary enhancement of SR Ca leak should reduce SR Ca load (opposite to observed). We conclude that PKA-dependent phosphorylation does not alter RyR2 binding of FKBP12.6, but enhances CaSpF secondary to PLB phosphorylation-dependent increase in SR Ca content.

Unlike F-FKBP12, wash-out of F-FKBP12.6 was extremely slow on removal of bath F-FKBP12.6 was extremely slow on removal of bath F-FKBP12.6, suggesting much higher affinity for F-FKBP12.6 (see below). Even rapamycin addition after F-FKBP12.6 equilibration only very slowly decreased fluorescence, without significant CaSpF alteration within 10 minutes. Thus, F-FKBP12.6 (but not FKBP12) inhibited resting RyR2 in situ, but cAMP-dependent PKA phosphorylation had no acute effect on FKBP12.6/12-RyR2 binding.

FKBP12.6/12 Binding Kinetics

Three methods were used to measure FKBP-RyR2 binding affinity and kinetics.

Steady-State K_d and B_max Measurement

To measure FKBP12.6 binding affinity in situ, we incubated permeabilized myocytes with different [F-FKBP12.6]. Figure 4A and 4B shows that F-FKBP12.6 binding is prevented by 20 μmol/L rapamycin. This also shows that F-FKBP12.6 fluorescence inside myocytes was almost the same as in the bath (providing quantitative control for analysis). Fluorescence intensity at Z-line, M-line, and cell average were plotted versus bath [F-FKBP12.6] (Figure 4D). The average fluorescence intensity represents the overall bound F-FKBP12.6 in myocytes. FKBP12.6 binding exhibited an apparent dissociation constant (K_d) in ventricular myocytes of 1 nmol/L at 100 nmol/L [Ca], higher affinity than a previous report, and the linear fluorescence-bath [F-FKBP12.6] relationship (Figure 4C) allowed calibration of the bound [F-FKBP12.6] in myocytes (Figure 4E). The apparent B_max (=1 μmol/L) is close to our previous measurements of total concentration of RyR2 monomers in rat ventricular myocytes, based on [3H]-ryanodine binding. Therefore, our data agree with RyR2 being the main high-affinity binding site for FKBP12.6, and with a stoichiometry of 4 FKBP12.6 per RyR2 tetramer. Note that additional soluble FKBP binding targets might be lost by myocyte permeabilization. Pretreatment with cAMP/OA did not affect steady-state affinity or B_max for FKBP12.6 (Figure 4E).

Figure 3. FKBP12.6-RyR2 association and resting RyR2 activity. A, Time course of FKBP12.6 (100 nmol/L) wash-in and influence of cAMP and rapamycin. B, Normalized FKBP12.6 and cAMP effects on CaSpF, FKBP12.6-RyR2 binding (n=25, 17), and SR Ca load (assessed by 10 mmol/L caffeine, n=6). Basal control CaSpF is 8.7±0.6 sparks (sec⁻¹ 100 μm⁻²). C, Normalized graph of rapamycin effects on CaSpF, FKBP12.6-RyR2 binding, and SR load (n=45, 10, 7). D through F, FKBP12.6, rapamycin, and cAMP effects on Ca spark amplitude, width (FWHM), and duration (FDHM).
and $k_{\text{off}}$, respectively). We used fluorescence recovery after photobleaching (FRAP) to investigate binding kinetics of FKBP12.6/12.27

Figure 5A shows a myocyte equilibrated with 1 nmol/L F-FKBP12.6, immediately after photobleaching 3 regions of interest (ROIs), and where striations have partially recovered at 30 minutes (right). This FRAP indicates replacement of photobleached F-FKBP12.6 with fresh F-FKBP12.6 from the bath, and depends on $k_{\text{on}}$ and $k_{\text{off}}$.28 Figure 5B shows exponential fits of FRAP with first order rate constant, $k_{\text{FRAP}}$. This provides an integrated measurement of $k_{\text{on}}$ and $k_{\text{off}}$ ($k_{\text{FRAP}}=k_{\text{on}} [\text{FKBP12.6}]+k_{\text{off}}$) and measurement at different [F-FKBP12.6] allows determination of $k_{\text{on}}$, $k_{\text{off}}$, and $K_d$ (Figure 5C).28 The apparent $K_d=1.3$ nmol/L for FKBP12.6-RyR2 agrees with our steady-state $K_d$ measurements. Rapa- mycin (20 μmol/L) completely prevented FRAP (Figure 5C). Inclusion of cAMP/OA caused no significant change in either $k_{\text{on}}$ or $k_{\text{off}}$, suggesting that cAMP-dependent PKA phosphorylation does not affect the binding kinetics of FKBP12.6 to RyR2 (see also Figure 6, C and E).

Diffusion of F-FKBP12.6 is $>1000$ times faster than FRAP and is thus not rate limiting (eg, extracellular FRAP in ROI3 was $<2$ s versus $\approx 1000$ s in the cell; Figure 5B). FRAP kinetics were also the same at the cell center or edge (ROI1 versus -2), suggesting that intracellular F-FKBP12.6 diffusion is not rate-limiting in our FRAP measurement (see also below).

FKBP12 has a much lower affinity for RyR2, which accelerates FRAP compared to FKBP12.6. This has two practical consequences. First, FKBP12 FRAP must be measured on a faster time scale, using line-scan mode.27 Second, F-FKBP12 diffusion may contribute to the measured FRAP, so it must be assessed. For line FRAP, we bleach a single line and monitor FRAP in that line at much lower laser power. As diffusion controls, we measured FRAP of F-dextran (molecular mass, 10 000 Da) in the myocyte (Online Figure IV), which recovered with a single time constant ($\tau=1.7$ sec $\pm 1/ k_{\text{FRAP}}$), and F-FKBP12 where binding was blocked by non-fluorescent FKBP12.6 (or rapamycin), giving $\tau=1$ sec. FRAP of FKBP12 (without blocking binding) was always best fit with a double exponential with a fast component ($\tau=1$ sec; taken as diffusion), and a slower component ($\tau=6$ to 10 sec) attributed to RyR2 binding. The slower $k_{\text{FRAP}}$ component was plotted as a function of [F-FKBP12] to extract $k_{\text{on}}$, $k_{\text{off}}$, and $K_d$ (Figure 5D). The $k_{\text{on}}$ for FKBP12 was similar to that of FKBP12.6, whereas $k_{\text{off}}$ was 150 times faster than FKBP12.6,
and consequently $K_d$ (210 nmol/L) was $\approx 150$ times higher than for FKBP12.6 and agrees with the steady-state $K_d$ measurements. Similar experiments were done in the presence of cAMP/OA, which again had no effect on $k_{on}$, $k_{off}$, or $K_d$ of FKBP12-RyR2 binding (see also Figure 6, D and E).

We previously showed that this cAMP/OA treatment in permeabilized ventricular myocytes leads to maximal RyR2 phosphorylation ($32^P$ incorporation) within 5 minutes. To test whether RyR2 phosphorylation increased here, we assessed RyR2 phosphorylation level at Ser2809 by quantitative western blot. Figure 7C shows results using isolated myocytes treated as in the confocal studies. Basal phosphorylation at Ser2809 is substantial (as in our previous reports). Permeabilization (before cAMP/OA exposure) produced a trend toward increase, but cAMP/OA treatment approximately doubled phosphorylation to what may be maximal phosphorylation.

**Binding Kinetics Measured by Continuous F-FKBP Wash-in/out**

Figure 6A shows the wash-in of 1 nmol/L F-FKBP12.6. Higher [F-FKBP12.6] greatly enhances the rate constant of wash-in, $k_{in}$ (eg, Figure 6B) and is stable during 30 minutes. Similar to FRAP analysis, $k_{in}$ depends on both $k_{on}$ and $k_{off}$ ($k_{in}=k_{on} [F-FKBP]+k_{off}$). After steady-state, the superfusate contained 33 nmol/L WT-FKBP12.6 (but lacked F-FKBP to prevent rebinding and have wash-out rate approximate $k_{off}$). The wash-out rate constant was no different in the absence of WT-FKBP12.6, suggesting that rebinding was negligible. The inferred $k_{on}$ and $k_{off}$ here are more variable, but agree with the FRAP measurements for both FKBP12 and 12.6 (Figure 6C through 6E and Online Table I). Wash-in/out experiments were also done after cAMP/OA exposure, and again binding characteristics were not altered for FKBP12 or 12.6 (Figure 6C through 6E and Online Figure V).

Remarkably, rapamycin did not alter F-FKBP12.6 (or F-FKBP12) wash-out kinetics (Figure 6C through 6E and Online Figure V). We infer that the rapamycin binding site on FKBP is only accessible when FKBP is not RyR2-bound. Thus, rapamycin acts by complexing with free FKBP12.6 to prevent its binding to RyR2, without interfering with RyR2-associated FKBP12.6.

**Endogenous FKBP12.6/12 Levels in Intact Rat Myocytes**

Given the extremely slow $k_{off}$ of FKBP12.6 from RyR2 ($\tau \gg 30$ minutes; Figures 5 and 6), we expect some endogenous FKBP12.6 to be retained after myocyte permeabilization, which could complicate our interpretation. So, it was surprising that the measured $B_{max}$ (Figure 4) matched the RyR2 monomer concentration in rat myocytes and that there was no discernable slow wash-in phase at high [F-FKBP12.6] (eg, Figures 3A and 6B) as endogenous FKBP12.6 gradually dissociated. This raises the possibility that endogenous [FKBP12.6] in myocytes is low compared to RyR2, giving a low fractional occupancy of RyR2 with FKBP12.6 in myocytes. That would also explain why RyR2 activity is virtually unaffected in FKBP12.6-KO mice. We measured amounts of endogenous FKBP12.6 and FKBP12 using three strategies.
First, we compared total available F-FKBP12.6 binding sites in WT versus FKBP12.6-KO mouse myocytes (as in Figure 6B with 100 nmol/L F-FKBP12.6). If WT mice exhibit substoichiometric occupancy of RyR2s by endogenous FKBP12.6, the apparent B\text{max} would be lower in WT versus FKBP12.6-KO mice. We took advantage of the very slow k\text{off} and fast k\text{on} of FKBP12.6, washing in saturating [F-FKBP12.6] (100 nmol/L) to rapidly reach steady-state binding (within 10 minutes), and minimize endogenous FKBP12.6 dissociation. F-FKBP12.6 had the same binding affinity for RyR2 in WT and FKBP12.6-KO myocytes, but B\text{max} was 20% smaller in WT versus KO mice (not significant; Figure 7A). Thus, at most ~20% of RyR2 sites are occupied with native FKBP12.6 in WT myocytes (consistent with 100 to 200 nmol/L cellular [FKBP12.6], assuming 0.5 to 1 μmol/L cytosol of RyR2 monomer).26

Second, we intentionally predepleted myocytes of native FKBP12.6 by preincubating intact myocytes with rapamycin (2 μmol/L) both before (1 to 2 hour, 37°C) and during permeabilization (followed by wash-out of rapamycin). In those studies, the apparent B\text{max} from F-FKBP12.6 experiments was only slightly increased by predepletion (10% to 15%, not shown).

Third, we directly measured endogenous FKBP12.6/12 in intact rat ventricular myocytes by western blot and FKBP concentration standards to establish a calibration curve (Figure 7A).
ure 7B). The first two lanes show a mixture of FKBP12/12.6 detected with antibodies that are FKBP12.6-specific (Laurent Vinet, INSERM U698, F-75018, Paris, France) and to a common epitope in both FKBP1s. FKBP12 and 12.6 migrate slightly differently allowing simultaneous detection. The average total FKBP12 was 6 ± 1.5 nMol/µg cell lysate, which implies 1.05 µMol/L cytosol of FKBP12 (based on 168 mg myocyte protein/mL cytosol[30]). We could not detect FKBP12.6 with either antibody in myocyte homogenates. Therefore the total amount of FKBP12.6 must be lower than the lowest detectable FKBP12.6 standard, ie, [FKBP12.6] ≤ 100 to 150 nMol/L cytosol. This agrees with the independent analysis above (at 100 nMol/L, only 10% to 15% of RyR2 monomers in the myocyte can possibly have FKBP12.6 bound), and the relative predominance of FKBP12 versus 12.6 observed by many investigators. Thus, FKBP12:FKBP12.6 is > 10:1 in cardiac myocytes, and [FKBP12] is roughly stoichiometric with RyR2 monomer concentrations.

Discussion

Using fluorescent FKBP and confocal microscopy of cardiac myocytes, we: (1) made the first in situ characterization of FKBP-RyR2 binding properties (on/off rates and $K_d$); (2) defined functional consequences of FKBP-RyR2 binding; (3) obtained further evidence that PKA-driven phosphorylation does not affect FKBP-RyR2 binding in the permeabilized myocyte setting; (4) estimated the amount of endogenous FKBP12.6 in permeabilized cardiac myocytes,31 which was similar to the FRET Bmax concentrations.

FKBP12.6/12 Binds Specifically to RyR2

We used several approaches to ascertain that RyR2 is the predominant myocyte FKBP target. First, the F-FKBP12/12.6 striation patterns overlap Di-8-ANEPs at T-tubules, where RyR2 is known to be located. Second, Ca sparks originated from F-FKBP labeled sites. Third, preincubation of myocytes with unlabeled FKBP12.6 completely abolished F-FKBP12 striations. Fourth, the FKBP $B_{max}$ measured equaled the concentration of myocyte RyR2 monomers. Fifth, we found strong and distinct FRET between F-FKBP12/12.6 donors and acceptor-labeled domains of calmodulin (F-CaM) in permeabilized myocytes,31 which was similar to the FRET reported in skeletal muscle SR and purified RyR1.24 Thus, in permeabilized cardiac myocytes, FKBP12.6 binds primarily to RyR2.

FKBP12.6/12 Binding Properties and Their Functions in Heart

A goal here was to measure FKBP-RyR2 interaction kinetics in situ in cardiac myocytes, because previous work used SR vesicles or cell lysates.2–4 Three independent methods demonstrated that FKBP12.6 binds RyR2 with very high affinity (≈ 1 nMol/L) and slow $k_{off}$, giving average dwell time of FKBP12.6 on RyR2 of ≈ 20 minutes, thus unlikely to modulate beat-to-beat. This, and the substoichiometric FKBP12.6:RyR2 and lack of FKBP12 effect on CaSpF, explains why FKBP12.6 overexpression can decrease resting Ca leak and consequently increases SR Ca content and Ca transients.11,32 Indeed, FKBP12.6 here inhibited CaSpF and amplitude, while increasing SR Ca load (although the effects were modest).

Rapamycin did not accelerate FKBP12.6 dissociation from RyR2, but instead, prevented rebinding of FKBP12.6 to RyR2. Thus, it is the slow off-rate of FKBP12.6 that determines the velocity of rapamycin effects on RyR2 activity. This clarifies various previous results where rapamycin produced either unaltered RyR2 activity (or heart function) or only small changes in spark duration or width, etc.7 However, lengthy rapamycin treatment indeed reverses FKBP12.6 effects in overexpression models,11,32 and in biochemical experiments.14,21

FKBP12 has a similar on-rate as FKBP12.6, but much faster off-rate and thus lower affinity. We could not detect effects of FKBP12 on RyR2 on CaSpF. This agrees with reports showing that coexpressing FKBP12.6 but not FKBP12 decreased Ca release triggered by an RyR2 activator (4-chloro-m-cresol) in CHO cells expressing hRyR2.16,18 However, FKBP12-deficient mice have normal skeletal muscles but severe dilated cardiomyopathy.33 Overexpression of FKBP12 in rabbit cardiomyocytes caused reduced CaSpF, and increased spark amplitude and duration, and Ca transients.17 Although these imply FKBP12 regulation of RyR2, a 48-hour rapamycin incubation was required to reverse the effects, whereas FKBP12 should dissociate from RyR2 within 5 minutes (Figure 2A). Thus indirect pathways cannot be excluded.34

FKBP12.6/12 Concentration and Its Physiological Role in Heart

We estimate endogenous myocyte FKBP12.6 and FKBP12 at 100 nMol/L and 1 µMol/L, respectively (Figure 6). Low total [FKBP12.6] and high affinity for RyR2, indicate that > 99% of myocyte FKBP12.6 is bound to RyR2, with subnanomolar free FKBP12.6. Our data also suggest that ≤ 20% of FKBP binding sites are occupied by native FKBP12.6 (Figure 7), so > 80% of RyR2 are available to bind FKBP12. Furthermore, the fast $k_{off}$ of FKBP12 means that permeabilizing myocytes allows endogenous FKBP12 to diffuse out ($r \approx 25$ s).

More than 80% of endogenous RyR2 are FKBP12.6-free. If FKBP12.6 binding were critical for normal RyR2 function (preventing SR Ca depletion and arrhythmias), then both normal myocytes and FKBP12.6-KO mice would be in an extremely precarious situation. Notably, female FKBP12.6-KO mice have no cardiac dysfunction, myocytes exhibit enhanced Ca transients and larger Ca sparks35 and bilayer experiments showed unaltered RyR2 activity.14 Although one group found exercise-induced arrhythmias and RyR2 channel hyperactivation in FKBP12.6-KO mice (only after exercise),9 another found no difference in stress-induced arrhythmias.14 This controversy is unresolved, but whether there is zero FKBP12.6 or 10% to 20% saturation of RyR2 (WT myocytes), SR Ca uptake and release can function relatively normally. Thus, although FKBP12.6 can inhibit diastolic RyR2 activity, the effect is moderate, and in vivo most RyR2 must achieve physiological stability somehow other than FKBP12.6 binding.

These permeabilized myocyte studies are most relevant to diastolic SR Ca release, which can be arrhythmogenic, and
SR Ca release during Ca current triggered release may be differently regulated. For example, PKA can accelerate the rate of SR Ca release and inactivation in myocytes when Ca current and SR Ca load are held constant.\(^3\)

**Relevance to HF: PKA-Driven Phosphorylation and FKBP12.6/12-RyR2 Binding**

Defective RyR2 regulation is linked to cardiac arrhythmias and contractile dysfunction in HF, via diastolic SR Ca leak via RyR2.\(^9,19,37\).\(^3\) Extensive publications by Marks and coworkers support the theory that, in HF, RyR2 hyperphosphorylation by PKA disrupts FKBP12.6-RyR2 binding, thereby enhancing SR Ca leak and unloading.\(^9,19,37,40\)

Some data from others support components of this model,\(^39,43\) but key aspects have been challenged by work showing either that RyR2 hyperphosphorylation fail to occur in HF, that PKA-dependent RyR2 phosphorylation does not cause FKBP12.6 dissociation, RyR2 activation, or SR Ca leak, or that FKBP12.6 does not alter RyR2 gating.\(^20,21,40\)

Our results cannot resolve this controversy, but provide new quantitative information about FKBP binding in the normal myocyte environment, with directly parallel functional data. In particular, we did not find any effect of PKA-dependent RyR2 phosphorylation on FKBP12.6 binding (measured 3 different ways). On the other hand, we detected significant diastolic RyR2 inhibition by FKBP12.6 (but not FKBP12). Our finding of low fractional saturation of RyR2 with FKBP12.6 in myocytes also means that even if FKBP12.6 association is reduced in HF, it could only occur at a minority of RyR2 (which would make those RyR2s like the others, in that respect). Of course, other effects in HF (eg, redox modulation or calcium/calmodulin-dependent protein kinase (CaM)KII-dependent phosphorylation\(^39,44,45\)) may alter RyR2 responsiveness to FKBP or PKA-dependent phosphorylation.

**Acknowledgments**

We thank Florentin Nitu for technical support; Drs Seth Robia, Sandra Despa, and Julie Bossuyt for critical comments; and Dr Susan Hamilton for providing FKBP12.6-KO mice.

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**Disclosures**

None.

**References**


What Is Known?

- Small intracellular immunophils FKBP12 and FKBP12.6 can bind to the cardiac ryanodine receptor (RyR2) and are thought to modulate RyR2 function as the SR Ca release channel.
- In some studies, protein kinase (PKA)-dependent RyR2 phosphorylation (especially in heart failure) has been reported to cause the dissociation of FKBP12.6 and consequent RyR2 leak, which could contribute to both reduced SR Ca content and arrhythmogenesis.
- Importantly, other studies have found comparable results, and the field remains controversial.
- FKBP12.6 is known to bind RyR2 with higher affinity than FKBP12, but there are no direct measurements of binding in the physiological cardiac myocyte environment.

What New Information Does This Article Contribute?

- The first direct measurements of FKBP12/12.6 binding kinetics with RyR2 in the adult cardiac myocytes using fluorescent FKBP and confocal microscopy. The $K_d$ for FKBP12.6 is $\sim$1 nmol/L and for FKBP12 is $\sim$200 nmol/L.
- Simultaneous measurements of RyR2 function (Ca sparks) and FKBP binding. FKBP12 had no functional effect, whereas FKBP12.6 inhibited Ca sparks and enhanced SR Ca content.
- Measurements of the in situ effects of PKA-driven RyR2 phosphorylation on FKBP association and RyR2 function (there was no detectable effect).

Novelty and Significance

- The endogenous myocyte concentrations of FKBP12 is 1 $\mu$mol/L and that of FKBP12.6 is $<100$ nmol/L, whereas the concentration of RyR2 is $\sim$1 $\mu$mol/L.

This study was designed in part to address the controversy in this field regarding the role of FKBP12/12.6 and PKA-dependent RyR2 phosphorylation and the relative lack of fundamental data about these issues, especially in the native cardiac myocytes. We developed a novel method to simultaneously measure FKBP binding to RyR2 and RyR2 function, allowing unique physiological insight into this interaction. We used three different ways to measure binding properties ($K_a$, $K_d$, and $K_m$), all of which gave comparable results. This work provides novel and important quantitative constraints. One major constraint is that the number of FKBP12/12.6 molecules in the normal myocyte is much smaller than the number of RyR2 monomers. Thus, only 10% to 20% of RyR2 in myocytes can be subject to FKBP12.6 modulation. Other major constraints are that binding of FKBP12.6 (but not FKBP12) inhibits Ca sparks, and that PKA-dependent RyR2 phosphorylation does not alter FKBP12/12.6 binding. Therefore, the increase in SR Ca content and Ca sparks by PKA must be mediated by other pathways (e.g., phospholamban phosphorylation and SR Ca-ATPase stimulation).
Kinetics of FKBP12.6 Binding to Ryanodine Receptors in Permeabilized Cardiac Myocytes and Effects on Ca Sparks
Tao Guo, Razvan L. Cornea, Sabine Huke, Emmanuel Camors, Yi Yang, Eckard Picht, Bradley R. Fruen and Donald M. Bers

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for
Kinetics of FKBP12.6 binding to ryanodine receptors in permeabilized cardiac myocytes and effects on Ca sparks
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Bradley R. Fruen, Donald M. Bers

METHODS
Expression, Purification, and Labeling of of FKBP Variants.
Single-cysteine FKBP cDNA constructs were derived by site-directed mutagenesis (QuikChange kit, Stratagene, La Jolla, CA) from the human sequences of FKBP12 and 12.6 (T14C/C22A-FKBP12 and T14C/C22A/C76I-FKBP12.6). Wild type and mutant FKBP variants were expressed and purified as previously described1.

The single-cysteine FKBP12 and 12.6 mutants were labeled using the sulfhydryl-reactive probes fluorescein-5-maleimide and Alexa Fluor 488 C5 maleimide, respectively (Invitrogen/Molecular Probes). Briefly, the FKBP sample (80 µM) was treated with TCEP (1 mM) for 1 hr in media containing MOPS (20 mM, pH 7.5), NaCl (150 mM). Fluorophore was then applied as powder, to a final concentration of 1.6 mM, DMF was adjusted to 20% by volume, and the reaction was allowed to proceed at room temperature, protected from light, with tumbling, for 4 hrs. To separate labeled FKBP from unreacted fluorophore, the sample pH was adjusted to 8.8 then loaded on a 1 mL DEAE Sephacel column (pre-equilibrated and developed with 20 mM TRIS pH 8.8). Fluorescently-labeled FKBP was recovered in the flow-through, while most of the unreacted label was retained on the column. Finally, the labeled FKBP sample was washed in an Amicon device (YM3 filters) using media containing MOPS (20 mM, pH 7.0) and NaCl (30 mM), then concentrated to 60-100 µM. The fluorescently-labeled FKBP's migrate on SDS-PAGE as a single band evidenced by UV illumination (Online Fig IA, UV). On coomassie-stained gels, labeled FKBP's show a slight up-shift relative to the unlabeled single-cysteine mutant (Online Figure IA, stained). Both unlabeled and labeled mutant FKBP12 & 12.6 recapitulate the difference in SDS-PAGE mobility characteristic between the wild type FKBP12 & 12.6 (Online Figure IA). The labeling efficiency was determined based on the FKBP concentration, as determined by gel densitometry, and on the concentration of bound fluorophore quantitated based on the extinction coefficient of labeled FKBP. The dye to protein ratio in the labeled FKBP12 samples used in this study ranged from 0.5 to 0.7.

Characterization of Labeled FKBP12 and FKBP12.6 in SR Vesicles
Circular dichroism (CD) spectra of FKBP variants were recorded from 200 to 250 nm (1 nm interval) in a JASCO J-710 spectrophotometer using a 1 mm path-length cell, at 25 °C with temperature regulation provided by a Peltier accessory. The FKBP samples (20 µM) were prepared in media consisting of 20 mM phosphate buffer (pH 7.0) and 30 mM NaF. Binding of fluorescently-labeled FKBP12 & 12.6 to cardiac SR membranes isolated from porcine ventricular tissue2 was characterized as previously described1 by exchange/binding isotherms, and by competitive inhibition of F-FKBP binding. Briefly, both assays involved 1.5-h incubations in 35°C Binding Media, which contained (in mmol/L): PIPES 20, KCl 150, GSH 5, EGTA 1, aprotinin/leupeptin 1 µg/mL, and BSA 0.1 mg/mL. To obtain the exchange and binding isotherms (Online Figure IC), samples containing Binding Media were supplemented with cardiac SR (0.2 mg/ml) and CaCl2 to achieve 30 nmol/L free [Ca2+]i, and were incubated in the presence of a range of F-FKBP12.6 and 12 concentrations, as indicated. To obtain the competitive inhibition profiles, Binding Media was supplemented with cardiac SR and CaCl2 as above, but samples contained F-FKBP12.6 (30 nmol/L) or F-FKBP12 (100 nmol/L) (Online Figure ID,
left and right, respectively) and were incubated in the presence of a range of unlabeled wild type FKBP12 and 12.6, as indicated. Following incubation, samples were spun down to remove unbound F-FKBP, and the membrane-associated F-FKBP was determined in a SpectraMax Gemini EM microplate fluorimeter (Molecular Devices, Sunnyvale, CA) from the 520 nm fluorescence intensity of the SDS-dissolved pellet (excitation at 488 nm), against standards prepared under the same conditions. Non-specific binding was determined in the presence of rapamycin (5 µmol/L). [3H] ryanodine binding studies were conducted as previously described. Briefly, Binding Media was supplemented with skeletal muscle SR [0.2 mg/ml; isolated from porcine longissimus dorsi, and stripped of FKBP12 by pretreatment with FK506 (2 µM)], recombinant mammalian calmodulin (1 µm), CaCl₂ to obtain the indicated free [Ca²⁺], 20 nM [³H]ryanodine, in the absence of added FKBP, and in the presence of WT-FKBP or F-FKBP, as indicated (Online Figure IE).

**Mouse Cardiac Myocyte Isolation**

Single mouse ventricular myocytes were enzymatically isolated as previously described and FKBP12.6-KO mice were kindly provided by Dr. S.L. Hamilton (Baylor College of Medicine, TX). All procedures were performed according to the Guiding Principles in the Care and Use of the Animals approved by the Council of American Physiological Society. Briefly, after anesthesia (isoflurane, 5%), hearts were excised and perfused (5 min, 37°C) with the minimal essential medium (MEM, GIBCO Life Technologies) gassed with 95% O₂/5% CO₂, before inclusion of collagenase B (0.5 mg/ml, Boehringer Mannheim) and protease (0.02 mg/ml, Sigma). Triturates were incubated (10 min, 37°C) in the same enzyme solution, washed and kept in 100 µmol/L Ca²⁺ MEM solution.

**Ca²⁺ Sparks in Permeabilized Cells using Confocal Microscopy**

Ventricular myocytes were superfused with relaxing solution containing (in mmol/L) EGTA 0.1, ATP 5, HEPES 10, potassium aspartate 150, MgCl₂ 0.25, and reduced-glutathione 10, at 23°C. The sarcolemma was permeabilized with saponin (50 µg/ml) for 30s. After permeabilization, myocytes were placed in internal solution composed of (mmol/L): EGTA 1; HEPES 10; K-aspartate 120; ATP 5; free MgCl₂ 1; reduced glutathione 10; free [Ca²⁺] 100 nmol/L (calculated using MaxChelator), creatine phosphokinase 5 U/ml; phosphocreatine 10; dextran (Mr: 40,000) 4%; Rhod-2 potassium salt 0.035; pH 7.2. Each cardiomyocyte was first superfused with 50 nM [Ca²⁺]i internal solution and baseline Ca²⁺ sparks were recorded. Then, the 1 µM cAMP and 1 µM OA were added to the solution to phosphorylate RyR via PKA. Ca²⁺ sparks were recorded by a laser-scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) as previously described. SR Ca²⁺ load was evaluated by Ca²⁺ transient amplitude upon rapid application of 10 mmol/L caffeine.

**Fluorescence Recovery after Photobleaching (FRAP)**

FRAP experiments were performed to estimate the kinetics of FKBP-RyR binding. Photobleaching of permeabilized myocytes incubated with F-FKBP12.6 was achieved by illuminating regions of interest with high laser power (80%) for 45 s. This protocol destroyed more than 95% of the fluorophores in regions measuring 14 x 4 µm, which was estimated by the % decrease of initial fluorescence intensity. Subsequently, striation pattern recovery was monitored by acquiring 30 images at intervals of 1-5 min for over 60 min. This recovery reflects the reversible binding of FKBP12.6 to RyR, which is expressed as following:

\[
\text{FKBP12.6} + \text{RyR} \xrightarrow{k_{on}} \text{FKBP12.6-RyR} \xrightarrow{k_{off}} \text{FKBP12.6} + \text{RyR}
\]

[F-FKBP12.6] vastly exceeds that of photobleached FKBP12.6 (i.e. nonfluorescent FKBP12.6); bath is an inexhaustible pool of F-FKBP12.6 that maintains constant [F-FKBP12.6] (unaffected by RyR binding). Therefore, we assume [F-FKBP12.6]_{bath} = [FKBP12.6]_{free}. Consequently, this reaction is reduced to a first-order reaction and the apparent rate constant of FRAP (k_{FRAP}) can be measured by fitting the recovery time-course to a single-exponential association function. k_{FRAP} is determined by
both $k_{\text{on}}$ and $k_{\text{off}}$ of FKBP12.6-RyR binding and can be expressed as $k_{\text{FRAP}} = k_{\text{on}} [\text{FKBP12.6}]_{\text{free}} + k_{\text{off}}$. Hence, we can measure the on/off rates of FKBP12.6-RyR binding by varying the bath [F-FKBP12.6]. Three regions of interest were chosen for each myocyte to ascertain that diffusion is not a rate-limiting step of FRAP.

The dissociation of FKBP12 from RyR2 is so fast that FKBP12 diffusion itself could potentially be a rate-limiting step in the FRAP measurement and thus complicate the analysis. Line-FRAP allows measurement of fast fluorescence recovery processes and we used it to separate the diffusion component from the binding/unbinding processes of FRAP for FKBP12. As shown in Online Figure IVA, the pre-bleach fluorescence level was obtained by illuminating the selected line (ROI) at low laser power 7% for 60 ms. The laser power was then increased to 100% for 120 ms to activate the photobleach protocol. Finally the laser power was switched back to 7% to record fluorescence recovery. The time course of F-FKBP12 FRAP was fitted to a double-exponential association function with a fast and a slow rate–constant. We hypothesize that the fast rate constant represents mainly the diffusion process, while the slow rate constant represents mainly the binding/unbinding process. We tested this hypothesis using three independent methods. One was to measure line-FRAP for fluorescein-dextran (MW=10 KDa, Invitrogen D1821), which consists of biologically-inert hydrophilic polysaccharides with a similar molecular weight as FKBP12. This compound does not display detectable specific binding to cytosolic components, as indicated by its homogenous distribution throughout the myocyte. Therefore, we fitted the time course of line FRAP for F-Dextran to a single-exponential association function and compared this time constant with the fast time constant of FRAP for F-FKBP12. As shown in Online Figure IV, the time constant of F-Dextran matches the fast time constant of F-FKBP12 FRAP for the same concentration of probe. In addition, the fluorescence recovery of F-Dextran is similar in the bath and cytosol (data not shown), suggesting similar diffusion in the bath and cytosol. The second method was to preincubate the myocyte with a saturating concentration of unlabeled FKBP12.6 (thus blocking the FKBP binding sites on RyR2), followed by incubation of F-FKBP12. The third method was to treat myocytes with a high concentration of rapamycin (20 µM) and F-FKBP12 (250 nM) concurrently, to block the binding of F-FKBP12 with RyR2. Between these different measurements, the F-FKBP12 binding/unbinding process of FRAP is eliminated, allowing us to resolve the diffusion process. Therefore, we fitted these FRAP time courses to a single exponential function and found similar time constants for both F-Dextran and the fast time constant for F-FKBP12 (Online Fig IVB-D). Taken together, it is therefore justifiable to assume that the fast time constant mainly reflects the diffusion component of FRAP for F-FKBP12 while the slow time constant reveals binding/unbinding processes.

**Western blots of myocyte samples**

For quantitation of endogenous FKBP12/12.6, the myocytes were immediately frozen in liquid N₂. For PKA phosphorylation experiments, myocytes were treated in three conditions: 1) intact myocytes without treatment to measure basal phosphorylation level; 2) permeabilized myocyte with saponin to estimate how permeabilization itself changes phosphorylation level; 3) saponin-permeabilized myocytes incubated with 1 µM cAMP/OA (cAMP from Sigma, and OA from Calbiochem) for 30 min. The reactions were terminated by adding 6x sample buffer (30% glycerol, 10% SDS, 600 mM dithiothreitol, 350 mM Tris/Cl (pH 6.8), trace of Bromophenol Blue) supplemented with NaF (50 mM) and protease inhibitors (P-8340, SIGMA, St. Louis, MO) and the samples were immediately frozen in liquid N₂. Western blot was carried out as previously described. Primary antibodies used were anti-RyR (MA3-925, from ABR, Golden, CO), anti-Ser²⁸⁰⁸ (from Badrilla, Leeds, UK), anti-FKBP12/12.6 (from ABR, Golden, CO) and an FKBP12.6-selective antibody (a gift from Dr. Laurent Vignet, France).

**Laser-scanning confocal microscopy**

Confocal images acquired using a Biorad Radiance 2100 laser-scanning confocal microscope equipped with an Argon ion laser, Green HeNe laser and with a Nikon Fluo x40 oil lens (N.A.=1.3). Rhod-2 (35 µM) and Di-8-ANEPPS were excited at 543 nm and emission was recorded using a 600 nm long pass filter. F-FKBP's were excited at 488 nm, and emission was recorded using 515/15 nm bandpass filter. All experiments were done at room temperature.
Statistics
Ca^{2+} sparks were analyzed as previously described using SparkMaster\(^8\). Ca^{2+} spark amplitudes were normalized to fluorescence baseline (F\(_0\)) as \(\Delta F/F_0\), duration was full-duration half-maximum (FDHM) and width was full-width half-maximum (FWHM). Data were expressed as mean ± SEM, and significance was evaluated by using Student's t-test or one-way ANOVA.

Online Table I. Summary of FKBP12.6/12-RyR2 binding properties in permeabilized rat ventricular myocytes

<table>
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<th></th>
<th>(k_{on}) (x 10(^6) s(^{-1}) M(^{-1}))</th>
<th>(k_{off}) (x 10(^{-3}) s(^{-1}))</th>
<th>(K_d) (nM)</th>
</tr>
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<tr>
<td></td>
<td>FRAP</td>
<td>Perfusion</td>
<td>FRAP</td>
</tr>
<tr>
<td>F-FKBP12.6</td>
<td>0.33 ± 0.08</td>
<td>0.18 ± 0.05</td>
<td>0.42 ± 0.07</td>
</tr>
<tr>
<td>+ cAMP/OA</td>
<td>0.37 ± 0.12</td>
<td>0.22 ± 0.01</td>
<td>0.44 ± 0.11</td>
</tr>
<tr>
<td>+ Rapa</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>N</td>
<td>3-10</td>
<td>3</td>
<td>3-10</td>
</tr>
<tr>
<td>F-FKBP12</td>
<td>0.29 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>61.1 ± 4.1</td>
</tr>
<tr>
<td>+ cAMP/OA</td>
<td>0.24 ± 0.12</td>
<td>0.10 ± 0.03</td>
<td>69.0 ± 3.6</td>
</tr>
<tr>
<td>+ Rapa</td>
<td>0</td>
<td>0</td>
<td>NA</td>
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<tr>
<td>N</td>
<td>5-10</td>
<td>5-6</td>
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References
SUPPLEMENTAL FIGURES

Online Figure I. A, SDS-PAGE of 2 µg wild type FKBP12 (WT-12.0), T14C/C22A-FKBP12 (14C-12.0), Fm-labeled T14C/C22A-FKBP12 (F-14C-12.0), wild type FKBP12.6 (WT-12.6), T14C/C22A/C76I-FKBP12.6 (14C-12.6), and AF488-labeled T14C/C22A/C76I-FKBP12.6 (F-14C-12.6) was performed in 15% TRIS-HCl precast minigels (Bio-Rad, Hercules, CA). Protein bands were evidenced by Coomassie dye staining (bottom), or UV illumination (top) of gel prior to staining; B, CD spectra of FKBP12.6 variants WT-12.6, 14C-12.6, and F-14C-12.6 (left), and of FKBP12 variants WT-12, 14C-12, and F-14C-12 (right); C, Cardiac SR exchange/binding isotherms of F-FKBP12.6 (left) and 12 (right) were obtained as described in Supplemental Methods; D, Competitive inhibition of F-FKBP12.6 (left) or F-FKBP12 (right) binding to cardiac SR by unlabeled WT-FKBP (●) and WT-FKBP12.6 (■) was measured as described in Supplemental Methods; E, [3H]ryanodine binding to skeletal SR was measured as described in Supplemental Methods in absence of added FKBP (○), and in presence of wild type (●) and labeled (○) FKBP12.6 (0.5 µM, left) or FKBP12 (2 µM, right).
Online Figure II. A, Confocal image of permeabilized myocytes after exposure to 1µM F-FKBP12 at 100 nM [Ca], (zoom=3); B, zoom= 8, red box is region of interest (ROI); C, Fluorescence intensity profile within ROI (dark green) and sinusoidal fit (light green); D, Line scan image of F-FKBP12 from same myocyte; E, simultaneous Ca spark measurement; F, overlap of D and E.
Online Figure III. A, Confocal image of a myocyte after staining in 1µM Di-8-ANEPPS (zoom=3); B, F-FKBP12.6 wash-in same myocyte; C, overlap of A and B; D, overlap of Di-8-ANEPPS and F-FKBP12.6 within the ROI delineated by the white box.
Online Figure IV. FRAP of Dextran and F-FKBP12. A, Time course image of a line FRAP experiment performed in a permeabilized rat ventricular myocyte incubated with 250 nM F-Dextran. First, prebleach scans along a selected line (ROI) are recorded for 60 ms at low laser intensity (7%); then, the ROI is photobleached for 120 ms at high laser intensity (100%), and fluorescence recovery recorded at different time intervals. The laser intensity was 0% between intervals during the recovery to minimize photobleach. Only the first 10 s of 50 s line FRAP experiment is shown. B, F-Dextran FRAP was fit with a single-exponential function ($\tau \approx 1.7$ s). Red dashed line represents the initial fluorescence intensity before photobleaching. FRAP is almost complete within 50 s, suggesting little immobile F-dextran. C. FRAP with 250 nM F-FKBP12 was best fitted to a two-exponential association function ($\tau_{\text{fast}} = 1.1$ s and $\tau_{\text{slow}} = 7.8$ s). Red dashed line represents the initial fluorescence intensity. D, Average data for F-FKBP12 and F-Dextran (n=6 each). The fast time-constant of F-FKBP12 is close to the time constant of F-Dextran, suggesting that the fast time-constant mainly reflects the diffusion component in the cytosol.
Online Figure V. Rapamycin does not significantly change the off-rate of FKBP12-RyR binding. A, Time course of washout of after equilibration with 1 nM F-FKBP12.6 (in the presence of 33 nM non-fluorescent FKBP12.6) in the absence or presence of 20 µM rapamycin (n=3 for each group, p=0.60). B, Effect of temperature on F-FKBP12.6 (100 nM) washout in the presence of 20 µM rapamycin (n=5-10 for each group). C, Time course of F-FKBP12 washout at RT, in the presence of 1 µM cAMP/OA or 20 µM rapamycin (n=6 for each group, P=0.1).