In Cardiomyocytes, Binding of Unzipping Peptide Activates Ryanodine Receptor 2 and Reciprocally Inhibits Calmodulin Binding

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**Rationale:** One hypothesis for elevated Ca\(^{2+}\) leak through cardiac ryanodine receptors (ryanodine receptor 2 [RyR2]) in heart failure is interdomain unzipping that can enhance aberrant channel activation. A peptide (domain peptide corresponding to RyR2 residues 2460-2495 [DPc10]) corresponding to RyR2 central domain residues 2460-2495 recapitulates this arrhythmogenic RyR2 leakiness by unzipping N-terminal and central domains. Calmodulin (CaM) and FK506-binding protein (FKBP12.6) bind to RyR2 and stabilize the closed channel. Little is known about DPc10 binding to the RyR2 and how that may interact with binding (and effects) of CaM and FKBP12.6 to RyR2.

**Objective:** To measure, directly in cardiac myocytes, the kinetics and binding affinity of DPc10 to RyR2 and how that affects RyR2 interaction with FKBP12.6 and CaM.

**Methods and Results:** We used permeabilized rat ventricular myocytes and fluorescently labeled DPc10, FKBP12.6, and CaM. DPc10 access to its binding site is extremely slow in resting RyR2 but is accelerated by promoting RyR opening or unzipping (by unlabeled DPc10). RyR2-bound CaM (but not FKBP12.6) drastically slowed DPc10 binding. Conversely, DPc10 binding significantly reduced CaM (but not FKBP12.6) binding to the RyR2. Fluorescence resonance energy transfer measurements indicate that DPc10-binding and CaM-binding sites are separate and allow triangulation of the structural DPc10 binding locus on RyR2 vs FKBP12.6-binding and CaM-binding sites.

**Conclusions:** DPc10-RyR2 binding is sterically limited by the resting zipped RyR2 state. CaM binding to RyR2 stabilizes this zipped state, whereas RyR2 activation or prebound DPc10 enhances DPc10 access. DPc10-binding and CaM-binding sites are distinct but are allosterically interacting RyR2 sites. Neither DPc10 nor FKBP12.6 influences RyR2 binding of the other. (*Circ. Res.* 2013;112:487-497.)

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synthetic peptide corresponding to a 36-residue stretch of the central domain (Gly3488-Pro3525) of RyR2. It has been shown that DPc10 can specifically and directly associate with the Ca2+ leakiness. A single point mutation in DPc10 (R2474S) destabilization can increase Ca2+ leakage, presumably because it binds to the Z-line in the CPVT mutants.19 DPc10 and related RyR2 peptides therefore may, serve as useful molecular probes to study the channel’s structure–function relationship. However, the details of DPc10 binding to RyR2, including affinity and kinetics, are still unknown.

In the present study, our goal was to characterize the binding of DPc10 to the RyR2 in the relatively intact environment of saponin-permeabilized rat ventricular myocytes. We used fluorescent probes, similar to our previous studies.14,21,22 Competitive inhibition of fluorescent DPc10 (F-DPc10) binding to RyR2 by non-fluorescent DPc10 (nonfluorescent [NF]-DPc10) showed that both binding affinity and function relationship. However, the details of DPc10 binding to RyR2 and its influence on CaM and FKBP12.6 binding and function. Furthermore, we used fluorescence resonance energy transfer (FRET) among fluorescent FKBP12.6, DPc10 and CaM to determine how DPc10 alters CaM and FKBP12.6 binding and to assess where the DPc10-binding site on RyR2 is in relation to CaM-binding and FKBP12.6-binding sites.21,22

Methods
Rat ventricular myocytes were isolated and permeabilized as previously described.23 All procedures were performed according to the Guiding Principles in the Care and Use of the Animals and were approved by the Council of American Physiological Society. An expanded Methods section can be found in the Online Data Supplement. Disrupting RyR2, DPc10, FKBP12.6, and CaM were labeled at specific sites with small fluorescence probes, similar to our previous studies.14,21 Competitive inhibition of fluorescent DPc10 (F-DPc10) binding to RyR2 by non-fluorescent DPc10 (nonfluorescent [NF]-DPc10) showed that both bind to RyR2 at the same site and same affinity (Online Figure IA). NF-DPc10 and F-DPc10 produce similar effects on Ca2+ sparks and SR Ca2+ content (Online Figure IB), confirming that F-DPc10 exhibits the same functional effect as NF-DPc10.

Results
Localization and Binding Isotherms of F-DPc10 in Permeabilized Myocytes
Figure 1A shows confocal images of saponin-permeabilized rat ventricular myocytes incubated with different concentrations of DPc10 labeled with 5-carboxyfluorescein at its N terminus (F-DPc10). Myocytes were exposed to 0.2, 0.5, and 5 µmol/L F-DPc10, with intracellular [Ca2+]i ([Ca2+]) set at 50 nmol/L. F-DPc10 fluorescence is highest at the Z-lines, where RyR2 is concentrated, forming a typical cross-striated pattern. The difference between fluorescence intensity at the Z-line (Fz) and M-line (FM) is taken to represent [F-DPc10] specifically bound at the myocyte Z-line. We calibrated the bound [F-DPc10] in permeabilized myocytes using the linear relationship between F-DPc10 fluorescence and bath [F-DPc10] (Figure 1B). In-cell F-DPc10–binding isotherms indicate an apparent dissociation constant (Kd) for F-DPc10 binding at the Z-line of 480±24 nmol/L; the maximal binding (Bmax), which reflects the concentration of F-DPc10–binding sites, was 1.59±0.03 µmol/L (Figure 1C).

This Bmax value for F-DPc10 is similar to our previous steady-state binding measurements of FKBP12.6 sites, which specifically bind to RyR2 in permeabilized myocytes with subnanomolar affinity. Thus,
we infer that RyR2 is the main target for F-DPc10 (see Discussion). To further test whether this Z-line–associated F-DPc10 represents RyR2-bound F-DPc10, we measured FRET between FKBP12.6 (known to specifically bind to RyR2 with subnanomolar affinity)14 and F-DPc10. Figure 2A shows confocal images of FRET between FKBP12.6 labeled with Alexa Fluor 568 as a donor (AF568-FKBP12.6) and different concentrations of DPc10 labeled with HyLite Fluor 647 (HF647) as an acceptor (HF647-DPc10). Donor (AF568-FKBP12.6) fluorescence at the Z-line was quenched by HF647-DPc10, but the M-line signal was not (Figure 2B). The apparent $K_d$ calculated based on enhanced acceptor fluorescence was $610\pm 61$ nmol/L, and the apparent $K_d$ calculated based on donor fluorescence quench was $450\pm 43$ nmol/L (Figure 2C). The donor quench measurement is less complicated (eg, by donor bleed-through), and consequently likely to be more accurate, yielding a $K_d$ value that is remarkably similar to that obtained in our direct measurements of F-DPc10 at the Z-line (Figure 1C).

**Binding Kinetics of F-DPc10 in Permeabilized Cardiac Myocytes**

To characterize DPc10-binding kinetics at Z-lines, we performed F-DPc10 wash-in (500 nmol/L) and washout experiments in permeabilized myocytes (Figure 3A). Association ($\tau_{\text{wash-in}}=79.0\pm 3.2$ minutes) and dissociation ($\tau_{\text{washout}}=149.8\pm 4.4$ minutes) were very slow compared with similar FKBP12.6 measurements.14 From the wash-in/washout measurements,
Figure 3. Kinetics of fluorescent DPc10 (F-DPc10) binding at the myocyte Z-line. A, Time course of F-DPc10 (0.5 µmol/L) wash-in and washout. B, Effect of [F-DPc10] (0.5 and 5 µmol/L) on \( \tau_{\text{washin}} \) and \( B_{\text{max}} \). Data are reported as mean±standard error.

we calculated the association and dissociation rates constants, \( k_{\text{on}} \) and \( k_{\text{off}} \), respectively, according to the following equation:

\[
k_{\text{wash-in}} = [\text{F-DPc10}]k_{\text{on}} + k_{\text{off}}
\]

where \( k_{\text{wash-in}} \) = \( k_{\text{on}} \) and \( k=1/\tau \) (s\(^{-1}\)). Accordingly, \( k_{\text{on}} = 202±20 \) (L mol\(^{-1}\) s\(^{-1}\)) and \( k_{\text{off}} = 0.11±0.01 \) (10\(^{-3}\) s\(^{-1}\)). Based on these values and \( K_d = k_{\text{off}}/k_{\text{on}} \), F-DPc10 binds at the Z-line with \( K_d = 580±69 \) nmol/L, consistent with the steady-state \( K_d \) measurements (Figure 1). We repeated this kinetic analysis using FRET between FKBP12.6 and DPc10, thus assessing the RyR2-specific DPc10 binding (Online Figure IIIA and IIIB). Both methods of detecting FRET (enhanced acceptor fluorescence and donor quench) showed slow association and dissociation rates similar to those in Figure 3 for direct detection of F-DPc10 binding at the Z-line. Based on these kinetic and affinity analyses, we infer that most of the Z-line–specific DPc10 binding is to RyR2. This is also consistent with \( B_{\text{max}} \) which would imply \( n = 1 \) DPc10 per RyR2 monomer.

We were intrigued by the slow \( k_{\text{wash-in}} \) and conducted measurements to further understand the basis of this slow association. We tested the hypothesis that at resting [Ca\(^{2+}\)], DPc10 access to its RyR2 binding site is sterically hindered. If the N-terminal and central domains are tightly zipped to each other, then this interaction may occlude the DPc10-binding site on the RyR2, thus limiting the \( k_{\text{on}} \) for DPc10. Alternatively, a limiting factor may be the rate at which DPc10 adopts a conformation that can bind to RyR2. To discern between these mechanisms, we determined the effect of [F-DPc10] on \( \tau_{\text{washin}} \).

If the small fraction of DPc10 in the right conformation limits the binding rate, then this interaction may occlude the DPc10-binding site on the RyR2, thus limiting the \( k_{\text{on}} \) for DPc10. To test this, we first monitored F-DPc10 wash-in at elevated \( \tau_{\text{washin}} \). Although it did increase \( B_{\text{max}} \) (Figure 3B). The same was seen when using FKBP12.6–DPc10 with \( 0.5 \) vs 5 µmol/L, HF647-DPc10 (Online Figure IIIA and IIIB). These results indicate that F-DPc10 association at its RyR2 binding site exhibits restricted access by a factor residing on RyR2 (eg, binding site opening or transitions from zipped to unzipped state).

In our working model, under resting conditions, the RyR2 closed state may be stabilized by the interaction between the N-terminal and central domain in the zipped state. We hypothesized that conditions that promote RyR2 opening might enhance the rate of unzipping and accelerate \( \tau_{\text{wash-in}} \) for F-DPc10. To test this, we first monitored F-DPc10 wash-in at elevated Ca\(^{2+}\) (500 nmol/L). However, the 13% faster mean \( \tau_{\text{wash-in}} \) was not significant (Figure 4A). Although 500 nmol/L Ca\(^{2+}\) can increase RyR2 opening, it does not prolong open time appreciably, and the latter might be important in the propensity for unzipping. Thus, we preincubated myocytes with ryanodine (100 µmol/L) plus caffeine (5 mmol/L), which are known to favor long RyR2 openings and were reported to cause RyR2 domain unzipping in HEK293 cells.25 Ryanodine + caffeine produced a 21% faster \( \tau_{\text{wash-in}} \) (\( P = 0.002 \); Figure 4B).

Figure 4. Effect of cardiac ryanodine receptors (RyR2) channel modulators on the kinetics of fluorescent DPc10 (F-DPc10) Z-line association. A, Time course of F-DPc10 (0.5 µmol/L) Z-line binding in internal solution containing low [Ca\(^{2+}\)] or high [Ca\(^{2+}\)] or after a 3-hour pre-equilibration with saturating [NF-DPc10] (2 µmol/L) in low [Ca\(^{2+}\)]. Data are reported as mean±standard error (SE). B, Time course of F-DPc10 (5 µmol/L) Z-line binding after a 3-hour pre-equilibration in internal solution containing ryanodine (100 µmol/L) and caffeine (5 mmol/L). Data are reported as mean±SE (n values on bars; a.u., arbitrary units).
with NF-DPc10 (and then NF-DPc10 washout with F-DPc10 present; Figure 4A). This treatment significantly accelerated F-DPc10 association by a factor of \( \approx 2 \) (Figure 4A). None of these treatments significantly altered \( B_{max} \) (Figure 4A and 4B). Assuming that \( k_{off} \) of NF-DPc10 is the same as for F-DPc10 and using Equation, the \( k_{on} \) is increased by 3.2-fold by unzipping as a result of prebinding of NF-DPc10 to the RyR2. Our working hypothesis is that the RyR2 open state may increase the probability of an RyR2 shifting to the unzipped state and may allow faster F-DPc10 wash-in. It also seems that the RyR2 open state (favored by caffeine–ryanodine) differs from the unzipped state (bound with DPC10).

**Cross-talk Between F-DPc10 and CaM or FKBP12.6 Binding**

**Effect of CaM and FKBP12.6 on DPC10 Binding at the Myocyte Z-Line**

Both FKBP12.6 and CaM bind to the RyR2 and can reduce channel opening, which might alter DPC10 binding. Figure 5A shows representative confocal images of FKBP12.6 (100 nM) and CaM (1 \( \mu \)M) effects on DPC10 binding, as detected after a 200-minute incubation with F-DPC10. Although pre-equilibration with saturating CaM (1 \( \mu \)M) greatly reduced F-DPC10 binding, pretreatment with FKBP12.6 (100 nM) did not alter F-DPC10 binding in permeabilized myocytes. Neither CaM nor FKBP12.6 pretreatment altered M-line F-DPC10 fluorescence (Online Figure IV). Figure 5B shows the time course of F-DPc10 wash-in with or without pretreatment with FKBP12.6 or CaM. Saturation of RyR2 with FKBP12.6 (100 nM) did not alter either F-DPc10 maximal binding (\( B_{max} \)) or \( \tau_{wash-in} \). In contrast, saturation of RyR2 with CaM dramatically reduced \( B_{max} \) for F-DPc10 and slowed DPC10 access to its binding site, as indicated by the large increase in \( \tau_{wash-in} \) (Figure 5C). We infer that CaM stabilizes the domain interaction between N-terminal and central domains in the zipped state and thereby may reduce DPC10 access to its binding site. To test for direct CaM–DPC10 interaction, we performed control FRET measurements between donor-labeled CaM and acceptor-labeled DPC10 in solution in the absence of RyR. The maximal FRET efficiency (<1%) ruled out direct CaM–DPC10 interaction.

Next, we asked whether RyR2 is activated by DPC10 and whether FKBP12.6 or CaM can prevent this. We assessed Ca\(^{2+}\) sparks in permeabilized myocytes perfused with internal solution containing 50 nM free Ca\(^{2+}\) plus 1 \( \mu \)M autacamide-2–related inhibited peptide (to inhibit CaMKII activity). Line-scan images were recorded after 3-hour incubations under control conditions and in the presence of 5 \( \mu \)M DPC10, with or without 1 \( \mu \)M CaM or 100 nM FKBP12.6 (Figure 5D). DPC10 robustly increased CaSpF vs control, an effect almost completely blocked by CaM (Figure 5E). However, CaSpF activation by DPC10 was only slightly decreased by FKBP12.6 (Figure 5E), and not decreased at all when normalized to SR Ca\(^{2+}\) content (Online Figure V). In DPC10-treated permeabilized myocytes, Ca\(^{2+}\) spark full width at half maximum and full duration at half maximum were significantly increased compared with control and decreased when pretreated with CaM (Online Table I).

Because CaSpF strongly depends on the SR Ca\(^{2+}\) content, we also measured SR Ca\(^{2+}\) content as the amplitude of caffeine-induced Ca\(^{2+}\) release (Figure 5E). In cells treated with DPC10 with or without FKBP12.6, the SR Ca\(^{2+}\) was significantly lower than under control conditions. In contrast, treatment
with CaM plus Dpc10 resulted in no significant decrease in SR Ca\(^{2+}\) content vs control. Thus, the increased CaSpF in the presence of FKBP12.6 plus Dpc10 cannot be secondary to increased SR Ca\(^{2+}\) content (which was in fact decreased). These results are consistent with a Dpc10-induced increase in RyR2 channel activity resulting from defective interaction between N-terminal and central domains. This also agrees with the lack of FKBP12.6 effect on F-Dpc10–binding kinetics (Figure 5B) and the potient inhibition of Dpc10 binding by CaM (which may promote the zipped state and inhibit Dpc10 access).

**Effect of Dpc10 on FKBP12.6 and CaM Binding in Permeabilized Myocytes**

To examine the converse influence that Dpc10 may have on FKBP12.6 and CaM binding to RyR2 in situ, we used fluorescent FKBP12.6 and CaM variants labeled with Alexa Fluor 488 or 568 (AF488 and AF568, respectively). These fluorescent proteins were added to saponin-permeabilized myocytes with or without pre-equilibration with saturating Dpc10 concentration. First, we found that AF488-FKBP12.6 at 1 nmol/L (near its \(K_d\)\(^{18}\)) forms a striated pattern that is not affected by preincubation with 5 nmol/L Dpc10 (Online Figure VIA and VIB). Thus, Dpc10 does not influence FRET between AF568-FKBP12.6 and AF568-34-CaM (acceptor in the N-terminal domain)\(^{21}\) at a [CaM] near \(K_d\) (20 nmol/L; Figure 6Ai). Using direct excitation at 543 nm (emission at >600 nm), we detected total CaM at the Z-lines (Figure 6Ai). We also did this with high [CaM] (500 nmol/L) that saturates RyR2 with CaM under control conditions (without Dpc10; Online Figure VIC). Figure 6B shows that pretreatment with Dpc10 significantly reduced CaM binding (at 20 nmol/L CaM) both at the RyR2 and overall at the Z-line, and by similar proportions. Even at high AF568-34-CaM levels (500 nmol/L), Dpc10-treated myocytes exhibited reduced CaM binding at the RyR2 (FRET and at the Z-lines) vs control. Thus, once F-Dpc10 binds to the RyR2 and decreases N-terminal–central domain interactions, it reduces the CaM affinity for RyR2. Taken together, these results show that Dpc10 and CaM binding to RyR2 are mutually inhibitory. To test whether Dpc10 and CaM bind at the same or nearby RyR2 sites, we measured FRET between CaM and Dpc10.

**FRET Between CaM and Dpc10**

We used a fluorescence donor probe (AF568) at the C-lobe of CaM\(^{21}\) (AF568-110-CaM) and HiLyte Fluor 647 (HF647) as the acceptor probe on the N terminus of Dpc10 (HF647-Dpc10). We used the acceptor photobleach approach with measurement of the resultant increase in donor (AF568-110-CaM) fluorescence in saponin-permeabilized myocytes (Figure 6C). To use this approach quantitatively, all acceptor (Dpc10) sites must be loaded so that all donors can participate in FRET.

Our results show that it is impractical to saturate RyR2 with both CaM and Dpc10 (Figures 5B and 6B). To overcome this challenge, we pre-equilibrated the myocytes with saturating HF647-Dpc10, thus loading all Dpc10-binding sites on RyR2. Then, when we added AF568-110-CaM (500 nmol/L), \(\approx 50\%\) of RyR2s had donor but all had acceptor, allowing quantitative analysis of enhanced donor fluorescence in acceptor photobleach. Figure 6C shows selective photobleach of HF647-Dpc10 (at 635 nm) in only the central region of the myocyte, and donor fluorescence was enhanced only in that region (lower left), indicating that donors and acceptors are within FRET range.

To rule out the possibility that there is energy transfer between a donor and multiple acceptors, we measured the relationship between donor fluorescence enhancement and acceptor photobleach and found a linear relationship (Figure 6D), which indicates a 1:1 stoichiometry for CaM–Dpc10 FRET. We interpret this result as clear evidence that the FRET efficiency (\(E\)) between AF568-110-CaM and HF647-Dpc10 reflects the proximity of 1 CaM to 1 Dpc10. \(E\) and donor–acceptor distance calculations are described in the Online Methods.

FRET efficiency between AF568-110-CaM and HF647-Dpc10 on 98.2%±0.2% acceptor photobleach was 0.89±0.01 (n=8). This corresponds to a distance of 53±1 Å (Figure 6E) based on \(R_0=75\) Å for the AF68-HF647 donor–acceptor pair. With an alternative donor probe (AF488), this time attached at the N-lobe of CaM, and the same acceptor (HF647) on Dpc10, we measured \(E=0.27±0.02\), which corresponds to an interprobe distance of 63±1 Å (Figure 6E). Thus, this result shows that the donor probes on CaM are 53 to 63 Å from the acceptor on Dpc10, suggesting that CaM and Dpc10 can simultaneously bind at distinct, yet nearby, sites within the RyR2 structure. This again favors an allosteric rather than competitive basis for the mutual inhibition seen between CaM and Dpc10 binding to the RyR2.

**FRET Between FKBP12.6 and Dpc10**

To gain further information about the topology of the Dpc10–binding site on RyR2, we used the location of FKBP12.6 as a reference point.\(^{21,27,28}\) FKBP12.6 was labeled at position 1421 with the fluorescent donor AF488 (AF488-FKBP12.6) or AF568 (AF568-FKBP12.6), whereas Dpc10 was labeled with the acceptor HF647. We used the same 2 methods to measure FRET in permeabilized myocytes. Figure 7A shows that when HF647-Dpc10 (5 μmol/L) was added to myocytes equilibrated with donor (50 nmol/L AF568-FKBP12.6), there was strong reduction in donor emission (560–620 nm) and simultaneous appearance of FRET in the acceptor emission channel (655–755 nm). Next, we monitored the increase in donor fluorescence after acceptor photobleach when both donor (AF568-FKBP12.6) and acceptor (HF647-Dpc10) were pre-equilibrated (Figure 7B). Figure 7B shows the increase in acceptor fluorescence before bleach and the increase in donor fluorescence after acceptor photobleach in only part of the myocyte, resulting in locally enhanced donor fluorescence. FRET between AF568-FKBP12.6 and HF647-Dpc10 was almost complete (Figure 7A and 7B), indicating close proximity between the donor and acceptor probes. To better gauge the FKBP12.6–Dpc10 distance, we used an alternative donor probe, AF488-FKBP12.6, and the same HF647 acceptor on Dpc10 (to reduce \(R_0\) for the FRET pair). Representative confocal images of donor quench and acceptor photobleach using AF488-FKBP12.6 as a donor are shown in Online Figure VIIA and VIIB.

To ensure that FRET between FKBP12.6 and Dpc10 accurately reflects interprobe distance, we performed several controls. As shown in Online Figure VII C, there was no significant...
Figure 6. The effect of DPc10 on calmodulin (CaM) and FK506-binding protein 12.6 (FKBP12.6) binding to cardiac ryanodine receptors (RyR2) in cardiac myocytes. A, Representative confocal image of the effect of DPc10 on AF568-CaM binding at the Z-lines (i, Ex=543 nm) and at the RyR2 detected by fluorescence resonance energy transfer (FRET) between AF488-FKBP12.6 (donor) and AF568-CaM (acceptor) (ii, Ex=488 nm). Myocytes were incubated with 5 nmol/L DPc10 (3 hours, 25°C) before adding CaM. B, Quantitative analysis of data from (A) for 20 and 500 nmol/L CaM. Data are reported as mean±standard error (SE). C, Confocal images illustrating FRET between AF568-110-CaM (donor) and HF647-DPc10 (acceptor) measured using the acceptor photobleaching method. Photobleached area is clearly delineated in the middle of the myocyte image. D, Dependence of AF568-110-CaM fluorescence intensity on the extent of HF647-DPc10 photobleach. Data are best fitted by a linear function (R² = 0.986), indicating that each donor participates in FRET with only 1 acceptor. E, Summary of FRET efficiency E and distances between AF568-110-CaM and HF647-DPc10, and between AF488-34-CaM and HF647-DPc10 derived from FRET. Data are reported as mean±SE.

Figure 7. Fluorescence resonance energy transfer (FRET) between FK506-binding binding protein (FKBP12.6) labeled with AF568 or AF488 (donor) and HF647-DPc10 (acceptor) in permeabilized cardiomyocytes. A, Confocal images showing FRET as the decrease in AF568-FKBP12.6 fluorescence (donor quench) on addition of HF647-DPc10. B, Confocal images illustrating FRET as the increase in AF568-FKBP12.6 fluorescence after photobleaching HF647-DPc10. Acceptor photobleach is clear in the center of the confocal myocyte image. C, Dependence of AF568-FKBP12.6 and AF488-FKBP12.6 fluorescence intensity on the extent of HF647-DPc10 photobleaching. Data are best fit by a linear function (R² = 0.966 for AF568-FKBP12.6, R² = 0.972 for AF488-FKBP12.6), indicating that each donor participates in FRET with only 1 acceptor. D and E, Summary of E and distances between AF568-FKBP12.6/AF488-FKBP12.6 and HF647-DPc10 based on FRET measured by donor quench and acceptor photobleach. Data are reported as mean±standard error.

difference in direct acceptor fluorescence intensity with or without equilibrated donors. Online Figure VIIID indicates that photobleach of the acceptor was essentially complete in both cases (AF488-FKBP12.6, 98.9%±0.3%; AF568-FKBP12.6, 99.4%±0.4%). We also checked the stoichiometry of donor and acceptor using the method shown in Figure 6D. Figure 7C shows that fluorescence of AF568-FKBP12.6 and AF488-FKBP12.6 depended linearly on HF647-DPc10 fluorescence during progressive bleach, indicating that each donor is coupled to a single acceptor.
The average FRET efficiency between AF568/488-FKBP12.6 and HF647-DPC10 was used to estimate the distance between FKBP12.6 and DPC10. The FRET efficiency between AF568-FKBP12.6 and HF647-DPC10 measured by the donor quench method was \( E=0.92\pm0.01 \) (n=31), whereas that measured by acceptor photobleach method was \( E=0.91\pm0.01 \) (n=19; Figure 7D), corresponding to a distance of 50±1 and 51±1 Å. For the shorter \( R_e \) pair (AF488-FKBP12.6 and HF647-DPC10) FRET, \( E \) by the donor quench was 0.52±0.03 (n=20), and \( E \) by acceptor photobleach was 0.51±0.01 (n=24; Figure 7D), corresponding to distances of 53±1 and 54±1 Å, respectively. Thus, remarkably similar results were obtained with 2 different donor–acceptor pairs and 2 different methods for measuring FRET (Figure 7E). According to our FRET results, bound DPC10 is near both FKBP and CaM, which implies that reciprocal inhibition of CaM and DPC10 binding to RyR2 occurs through an allosteric mechanism rather than competition for the same binding site. Combining information from CaM–DPC10 and FKB12.6–DPC10 FRET allows triangulation of relative positions on the RyR2 (see Discussion).

**Discussion**

We used fluorescent DPC10, FKB12.6, CaM, and confocal microscopy of permeabilized cardiomyocytes and found the following: (1) DPC10 access to its binding site is sterically hindered in resting (zipped) RyR2; (2) F-DPC10 wash-in kinetics provides a sensitive measure of the RyR2 unzipped state in permeabilized myocytes; (3) DPC10 and CaM binding to RyR2 are mutually inhibitory (via allosteric rather than competitive interaction); and (4) DPC10, CaM, and FKB12.6 are physically 50 to 60 Å from each other as vertices of an approximately equilateral triangle on RyR2.

**RyR2 Is the Main Target of DPC10 Binding at Z-Lines**

To assess DPC10 binding affinity and concentration at Z-lines, we used equilibrium and kinetic binding methods. Both methods (Figures 1C, 2C, and 3A) yielded similar \( K_d \) values (\( \approx 500 \) nmol/L) and a \( B_{\text{max}} \) value of 1.6 µmol/L, which agrees with the concentration of RyR2 monomers and FKB12.6 at the Z-line in rat ventricular myocytes.\(^{14,29}\) This \( B_{\text{max}} \) value is higher than our previous measurements of \( B_{\text{max}} \) of FKB12.6 (\( \approx 1 \) µmol/L), which binds very specifically (\( \approx 1 \) nmol/L) to RyR2.\(^{14}\) The reason for this difference is that for DPC10 (vs FKB12.6), the fluorescence between Z-lines is a higher fraction of that at the Z-line (Online Figure VIII), in part because of the much higher DPC10 concentration required to saturate RyR2. For this reason, we used the difference in Z-line vs M-line fluorescence (\( F_{\text{Z-line}} - F_{\text{M-line}} \)) to assess specific binding of F-DPC10 at the Z-lines. For FKB12.6, we used cell average fluorescence\(^{14} \) to measure \( B_{\text{max}} \) in myocytes. If we reanalyze fluorescent FKB12.6 binding as we did for F-DPC10 (using \( F_{\text{Z-line}} - F_{\text{M-line}} \)), then the \( B_{\text{max}} \) for FKB12.6 was 1.3 µmol/L, consistent with the \( B_{\text{max}} \) for F-DPC10. Furthermore, the kinetics and affinity of Z-line–associated DPC10 were almost the same as that of RyR2 specifically bound to DPC10 (Figures 1–3 and Online Figures II and III). We conclude that RyR2 is the main specific Z-line target for F-DPC10.

**Access of DPC10 to Its RyR2-Binding Site Is Restricted**

We found that both the wash-in and washout kinetics of F-DPC10 binding (\( k_{\text{wash-in}} \) and \( k_{\text{wash-out}} \)) are extremely slow (Figure 3A). The calculated \( k_m \) for F-DPC10 is \( \approx 1800 \)-fold slower than that we measured for FKB12.6 under similar conditions.\(^{14}\) This suggests either that DPC10 very slowly adopts a conformation that can bind RyR2 or that the DPC10-binding site on RyR2 becomes available only very slowly. The insensitivity of \( k_{\text{wash-in}} \) to 10-fold higher [F-DPC10] (Figure 3B) is most consistent with the latter interpretation, indicating that \( k_m \) is limited by RyR2 properties that restrict the access of DPC10 to its binding site. Further supporting this hypothesis, pretreatment with NF-DPC10 (Figure 4A) robustly increased \( k_m \) (\( \approx 320\% \)). We infer that the bound NF-DPC10 shifted RyR2 to the unzipped state, allowing better access and exchange with F-DPC10. The simplest explanation for this is that the unzipped state relaxes back to the zipped state slowly with respect to F-DPC10 binding, so that when an NF-DPC10 dissociates it is more rapidly replaced by F-DPC10 (before rezippering and greater steric hindrance returns). A second related possibility is that 1 DPC10 molecule may bind at 2 sites to RyR2 (one with higher affinity than the other). When saturated by NF-DPC10 in the unzipped state, F-DPC10 may gain access and compete with NF-DPC10 at the low-affinity site. Then, when NF-DPC10 slowly dissociates from the high-affinity site, F-DPC10 is already local and can reach steady-state more rapidly (as observed). These are not mutually exclusive or unique possibilities.

We also found that enhancing RyR2 open state by caffeine plus ryanodine hastened the F-DPC10 association (Figure 4B). However, these effects on F-DPC10 \( k_{\text{wash-in}} \) were small compared with that of prebinding NF-DPC10, despite the very much stronger RyR2 channel opening expected. This agrees with Liu et al,\(^{24}\) who reported that DPC10 more strongly unzips the N-terminal and central domains than did ryanodine plus caffeine. Thus, we suggest that the unzipped and open states differ, although unzipping may increase RyR2 opening and that the open state may enhance the unzipping transition and DPC10 access (Figure 8A).

**Relationship Between FKB12.6 and DPC10 Binding to RyR2**

FKBP12.6 has been found to quiet RyR2 channel opening,\(^{30}\) but this is an intensely controversial issue.\(^{13,31}\) and FKB12.6 may only inhibit pathologically leaky RyRs.\(^{11}\) Because >80% of the RyRs in the cardiomyocytes have no natively bound FKB12.6,\(^{14}\) adding saturating concentrations of exogenous FKB12.6 should decrease Cu\(^{2+}\) leak caused by DPC10-induced unzipping. Here, we found that FKB12.6 has no effect on either DPC10 binding (\( B_{\text{max}} \) or \( k_{\text{wash-in}} \)) or vice versa (Figure 5A and 5B), and it does not quiet the activating effect of DPC10 on the CaSpF (Figure 5E). This is similar to our previous myocyte studies, in which FKB12.6 had very minor effects on Ca\(^{2+}\) sparks.\(^{14}\) Taken together, these results suggest that DPC10 and FKB12.6 act through independent mechanisms to modulate RyR2 function.
Here, we used methods designed to monitor this effect and found that unzipping the RyR2 by treatment with saturating [DPC10] reciprocally inhibits CaM binding to RyR2 (Figure 6B). There are two possible explanations for this reciprocal binding inhibition: (1) DPC10 and CaM compete to bind at overlapping sites (orthosteric mechanism) or (2) the DPC10-binding and CaM-binding sites are separate but coupled in a mutually inhibitory interaction (allosteric mechanism).

To discern between these possibilities, we assessed whether CaM and DPC10 can coexist on RyR2. In Figure 6C, we show strong FRET between donor-labeled CaM and acceptor-labeled DPC10 at Z-lines, indicating that CaM-binding and DPC10-binding sites in neighboring regions are simultaneously occupied. This conclusion is further supported by FRET measurements using two different donor–acceptor pairs and two different labeling sites on CaM, which indicate distances of 63±1 and 53±1 Å between DPC10 and the N-lobes and C-lobes of CaM, respectively (Figure 6E). Furthermore, FRET between FKBP12.6 and DPC10 indicates a distance of 53±3 Å between the probes, which can be compared with the 67±5 Å distance between a donor at position 14 of FKBP12.6 and an acceptor at position 34 of CaM that we previously reported. Taken together, these results strongly support the conclusion that DPC10 and CaM bind at separate sites on RyR2, and these interact through an allosteric mutually inhibitory mechanism.

Our working hypothesis (Figure 8A) that merits further study is as follows. The resting zipped RyR2 does not readily allow DPC10 access to its site (Figure 8A i) and CaM binding at a different site may stabilize this zipped state (Figure 8A iii). We suppose that the RyR2 can transition spontaneously between the zipped and the unzipped states (Figure 8A ii and iv), but that the low probability at rest causes the slow but eventual access of DPC10 to its site. This transition may be favored when the channel is open (caffeine plus ryanodine) and also in pathological conditions (e.g., HF). Once the central domain–mimicking DPC10 gains access and binds, it stabilizes the unzipped state (Figure 8A ii) that reciprocally facilitates channel opening and inhibits CaM binding (Figure 8A iv).

### Topology of the DPC10-Binding Site on RyR2

Although our aim here was not a detailed mapping of the DPC10-binding site within the cryo-EM 3-dimensional structure of RyR2, our FRET measurements help narrow the range of possible locations. The location of FKBP12.6 and CaM on the RyR2 structure is known from cryo-EM structural analysis, and their relative positions agree with our previous FKBP-CaM FRET studies.

**Relationship Between CaM and DPC10 Binding to RyR2**

In myocytes containing a CPVT-linked RyR2 mutation, β-adrenergic stimulation decreases CaM binding at the Z-lines, and this effect is mimicked in healthy myocytes by treatment with DPC10. Here, we used methods designed to monitor CaM and DPC10 binding specifically at the RyR2 in myocytes, aiming to understand the structural basis of the inhibition of CaM-RyR2 binding by DPC10. One important finding in the present study is that saturating CaM binding at the RyR2 dramatically reduced F-DPC10 binding and Ca²⁺ spark activation (Figure 5), presumably by stabilizing the zipped RyR2 state.

Our novel FRET-based method allows direct assessment of CaM-RyR2 binding in the native cardiac myocyte environment (using FKBP12.6-CaM FRET). Using this method, we found that unzipping the RyR2 by treatment with saturating [DPC10] reciprocally inhibits CaM binding to RyR2 (Figure 6B). There are two possible explanations for this reciprocal binding inhibition: (1) DPC10 and CaM compete to bind at overlapping sites (orthosteric mechanism) or (2) the DPC10-binding and CaM-binding sites are separate but coupled in a mutually inhibitory interaction (allosteric mechanism).

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**Figure 8. Proposed model of the interaction between N-terminal and central domains, and localization of the DPC10-binding site in the 3-dimensional (3D) structure of cardiac ryanodine receptors (RyR2).**

A. **Kinetic results (Figures 2-4)** suggest that fluorescent DPC10 (F-DPC10) access to its binding site is controlled by interdomain interaction within RyR2. i, The F-DPC10 access is sterically hindered in resting normal RyR2 (zipped state). ii, Pretreatment of RyR2 with physiological, pharmacological, or disease-mimetic agents that promote unzipping increase the F-DPC10 association rate. iii, Calmodulin (CaM) inhibits the F-DPC10 binding to RyR2. iv, DPC10 binding to RyR2 inhibits CaM binding to RyR2.

B. **Localization of DPC10 in the 3D structure of RyR2.** Fluorescence resonance energy transfer (FRET) data between CaM and DPC10 (Figure 6) and between FK-binding protein (FKBP) and DPC10 (Figure 7) suggest that DPC10 binds near to, or within, the RyR handle domain, between FKBP12.6 and CaM. The transparent blue sphere is centered at the surface projection (opaque blue ball) of the mass center of the cryo-EM densities where the atomic structure of RyR domain 1-559 was previously delineated. The location of FKBP12.6 and CaM on the RyR surface defines possible locations of the DPC10 acceptor within the RyR 3D structure. In the top view, we note that the FKBP sphere intersects the clamp domain. The dashed black circles approximately delineate the cryo-EM densities where the atomic structure of the skeletal muscle RyR domain 1-559 was previously docked. In the side view, the intersection continues through the clamp but also through domain 3. The locus of the DPC10 should be approximately at the intersections between the spheres (green arrows) and the RyR surface.
proposed seems quite far from the most probable location suggested by the FRET results. DPC10 is expected to bind the RyR2 within a 150-kDa N-terminal segment, containing the first 600 residues that form a hot spot of pathogenic mutations. The high-resolution structure of domain I to 559 has been reported and authoritatively docked into cryo-EM densities forming a vestibule in the cytoplasmic headpiece of RyR (see dashed black circles in Figure 8B). A different view in Online Figure X shows that our triangulation puts DPC10 close to, but not exactly at, that location. More detailed FRET analysis to triangulate the F-DPC10 marker is expected to more precisely locate the interdomain contact site.

We represent the FKBP12.6, CaM, and DPC10 sites all on the same face of the RyR2 tetramer. We previously showed that this is true for the FKBP–CaM FRET pair, but we also tested whether the potential DPC10 site could be between CaM and FKBP sites on adjacent RyR2 faces. Online Figure IX shows that this possibility is implausible based on our FRET measurements.

**Relevance to HF**

Until now, methods to monitor local conformational changes occurring in the interacting regulatory domains of RyR have relied on a large fluorescence quencher (used in isolated SR vesicles) or on FRET between a yellow fluorescent protein inserted into the N-terminal region and a cyan fluorescent protein inserted into the central region of RyR2 (in HEK293 cells). In this study, we show how the F-DPC10 wash-in kinetics can be used in the more native environment of permeabilized cardiomyocytes to evaluate domain interaction between the N-terminal and central domains of RyR2. This could serve as a powerful and versatile investigative tool in preclinical and clinical studies with respect to the domain unzipping hypothesis. For example, the time course of F-DPC10 wash-in can be monitored in myocytes from failing hearts in zipping hypothesis. For example, the time course of F-DPC10 washing in can be monitored in myocytes from failing hearts in heart failure.

**Sources of Funding**

This work was supported by National Institutes of Health R01-HL092097 (to D.M. Bers and R.L. Cornea) and P01-HL080101 (to D.M. Bers), and by Banyu Life Science Foundation International (to T. Oda).

**Disclosures**

None.

**References**


What Is Known?

- A synthetic peptide DPc10, which is a part of the central domain (Gly2460-Pro2495) of the ryanodine receptor 2 (RyR2), can destabilize RyR2 function by interfering with domain interaction between central and N-terminal domains (namely, domain unzipping).
- Calmodulin (CaM) inhibits RyR2 channel activity at all [Ca2+]i (both at diastolic and elevated [Ca2+]i), indicating that CaM stabilizes the closed state of RyR2. Domain unzipping also can reduce the affinity of RyR2 for CaM.
- FK506-binding protein 12.6 (FKBP12.6) is reported to bind RyR2 tightly as an important regulator in RyR2 gating, but no direct effect of DPc10 on FKBP12.6 binding has been seen in sarcoplasmic reticulum vesicles.

What New Information Does This Article Contribute?

- Measurements of both CaM–DPc10 and FKBP12.6–DPc10 FRET suggest that CaM and DPc10 binding interact via an allosteric (rather than orthosteric) mechanism.
- The location of the DPc10-binding site on the 3-dimensional RyR structure was detected using FKBP12.6–DPc10 and CaM–DPc10 FRET, and is near the handle domain and far from the clamp domain of RyR.

Defects in domain interaction between central and N-terminal domains of the RyR2 can destabilize RyR2 channel gating and increase Ca leak. However, details of DPc10 binding properties (dissociation constant, association rate constant, and dissociation rate constant) to RyR2 and its effect on CaM and FKBP12.6 binding in native cardiac myocytes are unknown. Here, we show that DPc10 association to its binding site is slow because of steric hindrance in resting RyR2, DPc10 wash-in kinetics was shown to be a useful tool for detecting domain the unzipping in the native cardiac myocytes environment. We found that domain unzipping differs from channel opening state, although unzipped state enhances the open probability of the RyR2. CaM binding at RyR2, but not FKBP12.6 binding, stabilizes RyR2 in the zipped state (blocking DPc10 binding and RyR activation); however, once DPc10 gains access and binds, it causes RyR unzipping by inhibiting CaM binding. Furthermore, FRET measurements demonstrate that DPc10-binding and CaM-binding sites are distinct but interact allosterically.
In Cardiomyocytes, Binding of Unzipping Peptide Activates Ryanodine Receptor 2 and Reciprocally Inhibits Calmodulin Binding
Tetsuro Oda, Yi Yang, Florentin R. Nitu, Bengt Svensson, Xiyuan Lu, Bradley R. Fruen, Razvan L. Cornea and Donald M. Bers

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Expanded Materials and Methods

**Rat cardiac myocyte isolation**

Single ventricular myocytes were isolated from rat hearts as described previously. 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 enzymatic solution, washed and kept in 100 µM Ca2+ MEM solution.

**Domain peptide**

DPC10 peptides unlabeled and labeled with 5-carboxyfluorescein or HiLyte FluorTM647 were synthesized at AnaSpec (Fremont, CA). The DPC10 sequence is: 2460-GFCPDHKAAMVLFLDRVYGIEV-QDFLLHLEVGFLP-2495. A single-cysteine variant of the human FKBP12.6 isoform (T14C-C22A-C76I-FKBP12.6) was labeled with Alexa Fluor 568 maleimide as described previously.1-3 A single-cysteine CaM (T34C-CaM) was labeled with Alexa Fluor 568 maleimide as described previously.2,3

**Fluorescent labeling of single-cysteine mutants of FKBP12.6 and CaM**

A single-cysteine variant of the human FKBP12.6 isoform (T14C-C22A-C76I-FKBP12.6) was labeled using the thiol-specific maleimide derivatives of Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen), as described previously.2,3 Single ventricular myocytes were isolated from rat hearts as described previously.1 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 enzymatic solution, washed and kept in 100 µM Ca2+ MEM solution.

**Laser scanning confocal microscopy**

Confocal images were measured 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. FRET experiments between CaM and DPC10, and FKBP12.6 and DPC10 were performed using an Olympus FV1000 confocal microscope. All experiments were done at room temperature.

**Ca2+ sparks in permeabilized cells using confocal microscopy**

Myocytes were permeabilized with saponin (50 µg/mL) for 60 seconds and placed in internal solution composed of EGTA 0.5 mM, HEPES 10 mM, K-aspartate 120 mM, ATP 5 mM, MgCl2 1 mM, reduced glutathione 10 mM, and free [Ca2+]i 50 nM (calculated using MaxChelator), creatine phosphokinase 5 U/mL, phosphocreatine 10 mM, dextran 4% Fluor-4 0.025 mM, pH 7.2. Ca2+ sparks were recorded by a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) as previously described.1 Fluor-4 was excited at 488 nm and emission was recorded using 500/530 nm bandpass filter. To assess SR Ca2+ content, caffeine (15 mM) was rapidly perfused. Ca2+ spark were analyzed as using SparkMaster.5

**FRET measurements**

For the FRET between CaM and DPC10, and FKBP12.6 and DPC10, we used Alexa Fluor 568 attached at the C-lobe of CaM (AF568-110-CaM)2, Alexa Fluor 488 attached at the N-lobe of CaM (AF488-34-CaM), Alexa Fluor 568-, or Alexa Fluor 488-FKBPI2.6.4 (AF488-FKBPI2.6, AF568-FKBPI2.6) and HiLyte FluorTM647-DPC10 (HF647-DPC10) as a donor-acceptor pair. AF488-, AF568- and HF647- were excited with separate laser channels of 488 nm, 543 nm and 635 nm, respectively. Emission fluorescence intensity data were obtained at 505-605 nm for AF488-FKBPI2.6/AF488-34-CaM, 560-620 nm for AF568-FKBPI2.6/AF568-110-CaM and 655-755 nm for HF647-DPC10. We used two experimental approaches, (1) comparing the donor fluorescence intensities before and after equilibration with acceptor (donor quenching) and (2) monitoring the increase in donor fluorescence after acceptor photobleaching (acceptor photobleaching), to detect and measure FRET signals in the permeabilized cardiomyocytes.

For the donor quenching method, FRET is indicated by a decrease in the donor fluorescence at wavelengths 505-605 nm (AF488-FKBPI2.6) or 560-620 nm (AF568-FKBPI2.6). The FRET efficiency (E) was calculated according to:

\[
E = 1 - \frac{F_{DA}}{F_D},
\]

Where \(F_D\) and \(F_{DA}\) are the fluorescence intensities of the donor-only and donor-acceptor samples, respectively.

Complete acceptor (HF647) photobleaching was achieved by repeated scans of a defined area of the myocyte with the 635 nm laser at maximum power, for 60 sec. E was calculated according to:

\[
E = \left(\frac{I_{donor-post} - I_{donor-pre}}{I_{donor-post}}\right) \times 100\%.
\]

where \(I_{donor-post}\) and \(I_{donor-pre}\) are donor fluorescence intensities before and after acceptor photobleach. Donor-acceptor distances, R, were calculated from the equation:

\[
R = R_0 \left(1 - \frac{1}{E}\right)^{1/6},
\]

where the Förster distance, \(R_0\) is defined as the distance at which E=0.5. \(R_0\) is calculated from:

\[
R_0 = 9780 \left(\frac{Jn^4 \phi_D}{\epsilon\lambda}\right)^{1/6},
\]

where n is the refractive index of protein in aqueous solution (1.4), \(\kappa\) is the orientation factor (set to 2/3, corresponding to random orientation), \(\phi_D\) is the fluorescence quantum yield of the donor (0.92 for AF488, and 0.69 for AF5685), J is the normalized spectral-overlap integral of donor emission \(F_D(\lambda)\) and acceptor absorbance \(\epsilon(\lambda)\), and is calculated from:

\[
J = \int \frac{F_D(\lambda) \epsilon(\lambda) \lambda^4 d\lambda}{|F_D(\lambda)| d\lambda},
\]

by numerical integration using a Microcal Origin template. For the AF488-HF647 and AF568-AF647 donor-acceptor pairs we used \(\epsilon_{HF647}(652nm) = 250,000\) (mol\(^{-1}\) cm\(^{-1}\)) to calculate \(R_0\) values of 54 A and 75 A, respectively.
Statistics
Data were expressed as mean ± SEM, and significance was evaluated using student's t test or one-way ANOVA. A P-value below 0.05 was considered statistically significant.

References
6. Jonson I, Spence MTZ. Molecular probes handbook, a guide to fluorescent probes and labeling technologies. Life Technologies. 2010

Table I. Ca2+ spark characteristics in permeabilized cardiomyocytes

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Sparks</th>
<th>Peak (F/F0)</th>
<th>FWHM (µm)</th>
<th>FDHM (ms)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1761</td>
<td>0.499 ± 0.004</td>
<td>1.28 ± 0.01</td>
<td>55.7 ± 0.67</td>
</tr>
<tr>
<td>+DPC10</td>
<td>1467</td>
<td>0.475 ± 0.004*</td>
<td>1.34 ± 0.01*</td>
<td>58.5 ± 1.00*</td>
</tr>
<tr>
<td>+FKBP12.6/DPC10</td>
<td>1921</td>
<td>0.439 ± 0.002*</td>
<td>1.21 ± 0.01*</td>
<td>60.5 ± 0.73*</td>
</tr>
<tr>
<td>+CaM/DPC10</td>
<td>2195</td>
<td>0.467 ± 0.003*</td>
<td>1.23 ± 0.01*</td>
<td>56.9 ± 0.74</td>
</tr>
</tbody>
</table>

Supplemental Figures

Online Figure I. A, Detection by FRET, as EAF or quenching of donor fluorescence, of the competitive inhibition of F-DPC10 binding to RyR2 by NF-DPC10 in permeabilized myocytes. Cells were incubated with F-DPC10 (F, 1 µmol/L) or with equal concentrations (1 µmol/L) of F-DPC10 and NF-DPC10 (F+NF). B, Ca spark frequency and SR Ca content for control (C) myocytes, and after addition of NF-DPC10 (NF, 5µmol/L) or F-DPC10 (F, 5µmol/L). Data are reported as mean ± SE (n values on bars).
Online Figure II. Kinetics of HF-DPc10 binding, measured by FRET between F-FKBP12.6 and HF-DPc10. Wash-in and wash-out time course of FRET after addition of HF-DPc10 (0.5 µmol/L), detected as EAF (A) or as donor quenching (B). Data are reported as mean ± SE (n=4).
Online Figure III. Effect of DPc10 concentration on the wash-in rate, measured using FRET between F-FKBP12.6 and HF-DPc10. FRET was detected as EAF (A) or as donor-fluorescence quenching (B), at 0.5 µM (circles) and 5 µM (triangles) HF-DPc10. Data are reported as mean ± SE (n values on bars).

Online Figure IV. Influence of FKBP12.6 or CaM on F-DPc10 fluorescence at the M-line. Addition of FKBP12.6 or CaM does not significantly change the M-line fluorescence intensity of F-DPc10. Data are reported as mean ± SE (n values on bars).

Online Figure V. Ca spark frequency normalized to the SR Ca content for myocytes after addition of DPc10 (5µmol/L), DPc10 (5µmol/L) plus FKBP12.6 (100 nmol/L), DPc10 (5µmol/L) plus CaM (1 µmol/L). Data are reported as mean ± SE (n values on bars).
Online Figure VI.  A Confocal image of permeabilized myocytes after exposure to 1 nmol/L AF488-FKBP12.6 in the absence or presence of DPc10 5 µmol/L.  B. Average data for AF488-FKBP12.6 binding to Z-lines with or without DPc10.  C. Confocal FRET image between AF488-FKBP12.6 100 nmol/L and AF568-CaM 500 nmol/L to detect CaM at the RyR2 and total CaM at the Z-lines with or without DPc10.

Online Figure VII.  A. Representative image of AF488-FKBP12.6 quench following addition of HF647-DPc10.  B. Confocal images showing AF488-FKBP12.6 and HF647-DPc10 fluorescence before and after acceptor photobleaching from permeabilized myocytes.  C. Summarized data of acceptor (HF647-DPc10) fluorescence intensity with or without equilibrated donors which was normalized to without donor condition.  D. The extent of the acceptor photobleaching in both cases (AF488-FKBP12.6 and AF568-FKBP12.6).
Online Figure VIII. Plot profile of striated sarcomeric pattern shows both F-DPc10 (5 μmol/L) binding and AF488-FKBP12.6 (50 nmol/L) binding at Z-lines and M-lines.

Online Figure IX. Variant of Figure 7B to better illustrate the location of the DPc10 acceptor within the RyR cryo-EM map, as suggested by FRET measurements from FKBP and CaM donors. The CaM-centered sphere (blue, R = 58 Å) is flanked by two FKBP-centered spheres (red, R = 53 Å) – one on the same face as the shown CaM, and the other one on an adjacent face. Spheres are of radii calculated from FRET, which indicate the distance between the donors at FKBP or CaM and the acceptor at DPc10 (green arrowhead). However, the FKBP and CaM spheres on adjacent faces of the RyR are separated by more than 20 Å (distance of closest approach indicated by magenta arrow in Top View), suggesting that the DPc10 donor does not locate in domains 5 or 9.

Online Figure X. Sight-lines via galleries formed between the peripheral RyR domains 3, 8, 9, and 10, and the more central domains 2 and 4 allow viewing the HF647-DPc10 locus along the inside face of domain 3.