FK506-binding proteins 12 and 12.6 (FKBPs) as regulators of cardiac Ryanodine Receptors: Insights from new functional and structural knowledge

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ABSTRACT
Ryanodine Receptors (RyRs) are intracellular Ca\(^{2+}\) channels that mediate Ca\(^{2+}\) flux from the sarcoplasmic reticulum in many cell types. The interaction of RyRs with FK506-binding proteins (FKBPs) has been proposed as an important regulatory mechanism, where the loss of this interaction leads to channel dysfunction. In the heart, phosphorylation of RyR has been suggested to disrupt the RyR-FKBP interaction promoting altered Ca\(^{2+}\) signaling, heart failure and arrhythmias. However, the functional result of FKBP interaction with RyR and how this interaction is regulated remains highly controversial. Recently, high resolution structures of RyR have provided novel aspects to the ongoing debate. This review will discuss the most recent functional data in light of these new structures.

KEYWORDS
arrhythmias; CPVT; FKBP; heart failure; Ryanodine Receptor; SR Ca leak

Introduction
Ryanodine Receptors (RyRs) are Ca\(^{2+}\) channels expressed in the membrane of the sarco(endo)plasmic reticulum (SR/ER) of many cell types as 3 isoforms (RyR1, RyR2 and RyR3). Each channel is a homotetramer with a molecular mass greater than 2 \(\times\) 10\(^6\) Da, with all isoforms having a mushroom-like structure with a large cytoplasmic region and a smaller hydrophobic membrane-spanning region. RyR1 is enriched in skeletal muscle, RyR2 in cardiac muscle and RyR3 is expressed at low levels in many tissues.\(^1\)

The cardiac ryanodine receptor (RyR2) is responsible for releasing Ca\(^{2+}\) from the sarcoplasmic reticulum during excitation-contraction coupling (ECC). In this physiologic process the opening of RyR2 occurs in response to extracellular Ca\(^{2+}\) entry in a mechanism known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). However, RyR2-mediated Ca\(^{2+}\) release can also occur in the absence of extracellular Ca\(^{2+}\) entry. This is termed spontaneous SR Ca\(^{2+}\) release, or due to its dependence on SR Ca\(^{2+}\) content; store overload-induced Ca\(^{2+}\) release (SOICR). SOICR is a recognized trigger of life-threatening arrhythmias due to Ca\(^{2+}\)-dependent alterations in the membrane potential of cardiac myocytes.\(^2\)

Given its importance for normal cardiac function, it is not surprising that RyR2 channel function is highly regulated and can be affected by the interaction of RyR2 with different molecules such as ATP,\(^3\) Zinc,\(^4\) and a multitude of small regulatory proteins. Some of the most studied regulatory proteins are FK506-binding proteins (FKBPs), which are cis-trans peptidyl-prolyl isomerases that bind the immunosuppressant drugs FK506 (Tacrolimus) and Rapamycin.\(^5\) Two members of the FKBP family are expressed within mammalian cardiac myocytes: FKBP12 (12 KDa, also known as fkbp 1a or calstabin1) and FKBP12.6 (12.6 KDa, also known as fkbp 1b or calstabin2). These isoforms share 85% sequence homology and have similar 3-dimensional (3D) structure.\(^6\) In the heart, FKBP12 and FKBP12.6 can bind RyR2 at a stoichiometry of 4 FKBP per RyR2 tetramer.\(^7,8\) FKBP12 binds RyR2 with lower affinity than FKBP12.6 but has a higher cardiac expression level.\(^9\)

The role of FKBPs in regulating RyR2 function has been the focus of many studies and yet many results are inconsistent leading to multiple controversies in the field. The group of Marks and colleagues have proposed that the interaction of FKBP with RyR promotes...
The closed state of the channel and decreases the appearance of subconductance states. They have also proposed that Protein Kinase A (PKA)-dependent phosphorylation of RyR2 at S2808 leads to loss of FKBP12.6, leading to SR Ca\(^{2+}\) leak, which ultimately impairs contractility and promotes arrhythmias.\(^{11,12}\) The same group has reported increased phosphorylation of RyR2 in humans with Heart Failure (HF) as well as animal HF models.\(^{11}\) Therefore, they are strong proponents of the hypothesis that FKBP loss has a pathophysiological role in HF.

However, these findings could not be repeated by other groups, which has led to the regulatory function of FKBP being challenged. The major controversy is the role of FKBP12.6 in “stabilizing” the channel function, additionally the role of PKA-dependent phosphorylation at S2808 in FKBP association has also been questioned. In the light of the recent high resolution 3D structures of RyRs,\(^{13-17}\) we reflect on the proposed roles of FKBP in RyR regulation and the importance of RyR phosphorylation on the interaction of RyR2 and FKBP.

**What is the phenotype promoted by altered FKBP interaction with RyR2?**

Originally, FKBP12.6 was thought to be the only FKBP isoform capable of binding to RyR2.\(^{18}\) More recent studies have shown that both cardiac FKBP isoforms bind RyR2,\(^{9}\) but RyR2 has much higher affinity for FKBP12.6 than FKBP12.\(^{6}\) However, FKBP12.6 is found at a much lower abundance than FKBP12 in cardiac tissue.\(^{8}\) This suggests that although FKBP12's affinity for RyR2 is lower than FKBP12.6 the greater abundance may result in more FKBP12 bound to RyR2 than FKBP12.6. Indeed a recent study found that only 20% of RyR2 proteins are associated with FKBP12.6 in myocytes.\(^{19}\) This relatively low level of occupancy could explain why RyR2 is virtually unaffected in FKBP12.6-KO mice in one report\(^{20}\) and questions whether FKBP12.6 is necessary for the normal gating of RyR2 channels under physiologic conditions.

Much of our knowledge of the role of FKBP on RyR2 function and Ca\(^{2+}\) release comes from transgenic animal studies. FKBP12.6-null mice have been developed by several groups in different genetic backgrounds. Unfortunately, the phenotypes of the null animals in different background strains are dissimilar. FKBP12.6-null mice generated in a DBA genetic background have structurally normal hearts with normal sinus rhythm. However, these animals have an increase in arrhythmogenic activity when stressed.\(^{21}\) In contrast, FKBP12.6-null mice developed in the 129S\(^{2}\)Ev background showed gender specific adult cardiac hypertrophy\(^{22}\) but no exercise-induced arrhythmia.\(^{20}\) Despite the difference in phenotype both models displayed enhanced Ca\(^{2+}\) spark frequency suggesting that loss of FKBP12.6 can alter RyR2 function in situ. Why in one model this is arrhythmogenic and another not is uncertain, but potential differences in Ca\(^{2+}\)-voltage coupling between both strains could be a possible explanation.

In addition to ablation of FKBP12.6, its role in RyR2 regulation has also been studied through the overexpression. In rabbit cardiac myocytes, FKBP12.6 overexpression decreases the occurrence, amplitude and duration of Ca\(^{2+}\) sparks, which in turn enhances Ca\(^{2+}\) transients by increasing SR Ca\(^{2+}\) content.\(^{23-25}\) The effect of FKBP12.6 overexpression on susceptibility to arrhythmias has been examined in a conditional FKBP12.6 overexpression mouse model. Here, the increase in FKBP12.6 was effective at preventing triggered ventricular tachycardia. However, whether this was simply due to the stabilization of RyR2 is difficult to ascertain as the increase in FKBP12.6 expression resulted in changes in L-type Ca\(^{2+}\) channel current (ICaL) and Na\(^{+}/Ca^{2+}\) exchanger (NCX) activity, either of which could also alter the susceptibility to arrhythmia,\(^{26}\) with no change in SR Ca\(^{2+}\) load.

FKBP12 has also been extensively studied in animal models. FKBP12-null mice experience severe dilated cardiomyopathy with most dying pre-term.\(^{27}\) However, whether the dramatic changes in cardiac structure can be attributed to loss of FKBP12 from cardiac myocytes has been questioned in subsequent studies.\(^{28,29}\) Cardiac myocyte specific FKBP12 overexpression has been studied in mice using the α-myosin heavy chain promoter. The animals showed a reduction in Na\(^{+}\) current (INa\(^{+}\)) with abnormal cardiac conduction and repolarization, which was associated with a 38% of mice dying suddenly at 4–6 weeks of age.\(^{28}\) Consistently, FKBP12 conditional knockout hearts showed an increase in INa\(^{+}\), suggesting that FKBP12 is an important regulator of INa\(^{+}\), at least in chronic models. On the other hand, the acute overexpression of FKBP12 in adult rabbit ventricular myocytes (using adenoviral transduction) showed a...
reduction in the gain of ECC and lower Ca\(^{2+}\) spark frequency, suggesting that FKBP12 reduces RyR2 sensitivity to cytosolic Ca\(^{2+}\). 30

**Effect of FKBP on RyR2 channel function**

A possible complication to understand the role of FKBP12 and FKBP12.6 in human hearts is that the expression of each variant differs significantly between different species. 31 Jeyakumar et al. 9 have suggested that FKBP12 binds to RyR2 in many species but may not bind canine RyR2. In other species including rabbit and human, the affinity of FKBP12 for RyR2 is appears to be 7 times lower than for FKBP12.6; therefore it is conceivable that the relative importance of the FKBP12-RyR2 interaction varies between species and may only be physiologically relevant in some. Interestingly, Peng et al. 16 have shown that the FKBP12 binding site is conserved in canine RyR2, therefore further work comparing high resolution local structures of canine and rabbit RyR2 is required to elucidate the apparent species dependent differences in FKBP association.

Given the apparent species dependent differences, single channel functional data are important to determine the effect of different levels of FKBP occupancy on RyR2. Unfortunately, data from RyR single channel recording yield contradictory results. The group of Marks and colleagues have repeatedly shown that FKBP12 can stabilize RyR1 function 32 and FKBP12.6 can stabilize RyR2 function. 11,21 They found that dissociation of FKBP12 from RyR1 or FKBP12.6 from RyR2 significantly increases the gating frequency of RyR, manifested as reduced open and closed times. 10,11 Interestingly, others show that while FKBP12 appears to stabilize RyR1 neither FKBP12.6 nor FKBP12 affects RyR2 function. 8,33 A possible factor confounding the different reports is the choice of compound used to dissociate FKBP from RyR2. Compounds with a macrocyclic lactone ring structure, such as Rapamycin or FK506 have been suggested to exert direct effects on RyR, independent of FKBP association. 20,34,35 However, other approaches such as mutation of FKBPs sites necessary to bind RyRs have been used by Marks group to support the stabilizing effect of FKBPs.

More recently, the roles of FKBP isoform on RyR regulation have become even more perplexing. In contrast to most of the field the Sitsapesan laboratory found that RyR1 activity can be increased by FKBP12.6 and RyR2 activity can be increased by FKBP12. 36,37 Specifically, they showed that FKBP12 increases open probability of RyR2 by sensitizing the channel to cytosolic Ca\(^{2+}\), while FKBP12.6 is a partial agonist of RyR2 with an extremely low efficacy. In this scenario, rather than a direct stabilization of the channel, an increase in FKBP12.6-RyR2 binding competes with FKBP12 at the same binding site and blunts the activation of RyR2 promoted by FKBP12. 37 Why this contradictory result was obtained is unclear but it is possible the answer may lie with the levels of FKBP12 present. Galfre et al. 37 used 1 \(\mu\)M FKBP12 (a concentration close to endogenous levels) 33 whereas others use overexpression of at least 2-fold or added it exogenously. While unlikely, it is possible that low concentrations of FKBP12 are sufficient to dissociate FKBP12.6 thus activating the channel, whereas high levels saturate the FKBP binding sites inhibiting the channel.

As opposed to altering the activation of the channel we recently demonstrated that both FKBP12 and FKBP12.6 mediate early termination of Ca\(^{2+}\) release leading to a reduced magnitude of Ca\(^{2+}\) release in each spontaneous release event. 38 These results are consistent with findings of others showing that overexpression of FKBP12.6 protects against cardiac arrhythmias by reducing Ca\(^{2+}\) spark amplitude, 23,24 and another demonstrating that exogenous FKBP12.6 reduces the frequency, amplitude and duration of Ca\(^{2+}\) sparks in permeabilized cardiac myocytes. 19 Also consistent with the role of FKBPs on SR Ca\(^{2+}\) release termination, it has been shown that the termination threshold of SR Ca\(^{2+}\) release is reduced in heart failure, a model where FKBP dissociation from RyR2 has been proposed. 39 Compared to FKBP12.6, the functional effect promoted by FKBP12 was less pronounced in our model (human embryonic kidney; HEK293 cells expressing RyR2). 38 This is consistent with the smaller apparent decrease in spark amplitude and width observed by others in permeabilized cardiac myocytes. 19

**Role of RyR phosphorylation on FKBP-RyR interaction**

One of the most studied post-translational modifications of RyR2 is phosphorylation. To date 3 phosphorylation sites have been identified: S2808, S2814 and
S2030. It is generally accepted that S2808 is the target of PKA and S2814 the target of Ca²⁺ calmodulin kinase II (CaMKII). However, the exclusivity of PKA for S2808 is questioned as some reports show both CaMKII and PKG are also able to phosphorylate this residue.⁴⁰-⁴² PKA has also been shown to be responsible for S2030 phosphorylation. S2030 has been proposed to be the primary site of regulation of RyR2 by PKA.⁴³ However, this finding is not universally accepted. Here the contrasting findings could be due to the sensitivity and specificity of the antibody used to study S2030.⁴⁴

The physiologic and pathological significance of RyR2 phosphorylation remains controversial. Marks and colleagues propose that S2808 phosphorylation triggers FKBP12.6 dissociation. As described above, they show that this promotes a gain of function leading to SR Ca²⁺ leak.¹¹,⁴⁵ However, consistent with the role of FKBP1s themselves, these findings are not supported by those of others. Several groups have shown that PKA-dependent phosphorylation of RyR2 does not promote FKBP12.6 dissociation from RyR2¹⁹,⁴⁶,⁴⁷ nor have any impact on Ca²⁺ sparks.⁴⁸ Further complicating the potential effect of PKA is the second potential phosphorylation site; S2030. Chen and colleagues indicate that S2030 and not S2808 responds to β-adrenergic stimulation suggesting it is the major site of PKA mediated regulation of RyR2. Additionally, they show that phosphorylation at either S2030 or S2808 has no impact on the association between RyR2 and FKBP12.6.⁴³,⁴¹

The role of phosphorylation is further muddied by results obtained from animal models. Marks and colleagues were the first to create a mouse where serine at 2808 was mutated to alanine (S2808A). This mutation prevents phosphorylation of this site. Initial studies showed that deletion of this site did not disturb normal cardiac function. However, the animals were protected from developing HF following myocardial infarction (MI), and from arrhythmias induced by phosphodiesterase inhibition.⁴⁵ In agreement with the animal phenotype, reconstituted RyR2 channels from S2808A mice did not display an elevated open probability after MI in contrast to those prepared from wild-type (WT) mice. Subsequently, Valdivia and colleagues generated a second S2808A mouse. This mouse was generated in the Sv129/C57Bl6 background as opposed to that created by Marks in the C57Bl6 background. Unlike the effect of serine ablation in the Marks model when these animals were subjected to HF using pressure overload (transverse aortic constriction), no cardio-protection was conferred.⁴⁹ Furthermore, no substantial differences could be observed between reconstituted WT and S2808A channel kinetics. Excitation-contraction coupling, Ca²⁺ sparks and waves were also compared between WT and S2808A and again no significant differences were found. This led Valdivia and colleagues to question the importance of S2808 on cardiac function. However, it was postulated that the dramatically different findings could be due to the use of different genetic background (C57Bl6 for Marks’s mice and Sv129/C57Bl6 for Valdivia’s mice). To address this, Valdivia’s group has recently generated a S2808A mouse in the same C57Bl6 background as used by Marks. In their hands this mouse yielded the same result as their own previous findings, leading them to discard differences in genetic background as an explanation for opposing results between the 2 groups.⁵⁰

In addition, Marks and colleagues have suggested that dissociation of FKBP12.6 from RyR2 and associated increase in channel function may be more complex and require oxidation and S-nitrosylation in concert with S2808 phosphorylation.⁵¹,¹² However, whether these modifications alter RyR2 function via FKBP12.6 dissociation is questionable as we and others have recently reported the direct effect of oxidation on RyR2 function and a lack of oxidation-induced FKBP12.6 dissociation.⁵²,⁵³

Effect of RyR mutations on FKBP-RyR interaction

Mutations in RyR2 have been linked with an arrhythmogenic condition known as Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) which manifests as exercise-induced bidirectional ventricular tachycardia. To date >150 mutations have been identified which are preferentially clustered into 4 regions.² One of the early studies to provide a mechanistic insight into how these mutations lead to arrhythmia was performed by Wehrens et al. who proposed that catecholaminergic hyperphosphorylation of RyR2 combined with the CPVT phosphorylation leads to dissociation of FKBP12.6.²¹ In turn, this loss of FKBP12.6 destabilises the channel resulting in an increase in Ca²⁺ leak. This finding was partially supported by work performed in HL-1 cells expressing several different CPVT mutations. In HL-1 cells, treatment with
isoproterenol significantly but equally increased the phosphorylation of WT and mutant channels and this led to dissociation of FKBP12.6. The level of dissociation was equivalent between WT and CPVT mutant channels suggesting a phosphorylation based effect rather than mutation effect.54 However, as described above, we and others have been unable to observe dissociation due to phosphorylation with or without CPVT mutations.55-57 Interestingly, although we have previously shown that CPVT mutations do not dissociate FKBP12.6 or alter the occurrence of SOICR we recently demonstrated that CPVT mutations abolish the ability of FKBP12.6 to reduce the magnitude of SOICR.38 This suggests that even in the absence of dissociation CPVT mutations may alter the way in which FKBP12.6 regulates RyR2 function.

Localization of the FKBP-binding domain in the RyR structure

Given the contentious nature of the effect of FKBP on RyR2 function it is perhaps unsurprising that the mechanism of interaction of FKBP with RyR2 is also debated. As described earlier, the very large size of the cytosolic domain of RyR2 makes identifying the site of interaction challenging especially given the, until recently, lack of high resolution structural information.

The N-terminal cytoplasmic assembly component of RyR is formed by ~ 85% of the ~ 5000 amino acids in the sequence of the protein.58 The cytosolic cap consists of multiple globular domains, interspersed by α-helical regions which form scaffolds for other domains. Between these, there are 3 SPRY domains (named after “SP1A kinase and Ryanodine Receptors” where they were first discovered)59 and 2 tandem repeats (repeat 1–2 and 3–4, the last considered as the phosphorylation hot spot).

Prior to structural information multiple groups attempted to identify the FKBP binding site using mutagenesis and protein-protein interaction studies. In the early 2000s, Marks and colleagues identified a valine residue (V2461) as being critical for FKBP12 interaction with RyR1.32 In keeping with the importance of this region, they also generated small fragments of RyR2 and proposed that isoleucine and proline residues (I2427 and P2428) form a hydrophobic-binding site for FKBP12.6,11 but a requirement for these residues was not examined in full-length RyR. The importance of these findings was challenged when Chen and colleagues mutated the proposed binding site of FKBP12.6 (I2428Q/P2428A) and found that the ability of FKBP12.6 to co-immunoprecipitate RyR2 was unaltered.60 Using partial RyR2 fragments spanning the length of RyR2, they found that deletion of amino acids between 305–1937 abolished FKBP12.6 binding. A subsequent study refined this to a region between 1815–1855.61 Later, Zissimopoulos and Lai confirmed the work of Chen by reporting a requirement for the N-terminus of RyR2 but not the central region. Interestingly they also identified an additional C-terminal region of RyR2 as interacting with FKBP12.6 with a low affinity. They showed that this region was specific for FKBP12.6 (no FKBP12 interaction was observed) and appeared to require a correctly folded protein.62

The recent surge of structural studies of RyR2 has led to a revolution in our knowledge of how the channel functions and is regulated. Early cryo-electron microscopy (cyro-EM) work inserting GFP into a site proximal to the FKBP12.6 binding site proposed by Chen (T1874-GFP) suggested that FKBP binds within Domain 9 in the ‘clamp’ structures which form the corners of RyR261 (Fig. 1). More recently crystal structures of the SPRY1 and tandem repeat domains combined with molecular docking onto high resolution cryo-EM structures of RyR1 have confirmed this location.63 Although the location is broadly the same, Yuchi et al. suggest that a

Figure 1. Schematic of FKBP interaction with RyR. FKBP is shown in red; SPRY1 in orange, SPRY 2 in blue and SPRY3 in green. Dotted line indicates the position of domain 9. View is from the cytosolic side of the channel.
SPRY1 loop (residues 650–844 in RyR2 and 639–833 in RyR1), which corresponds to Domain 9, is a major FKBP binding determinant (Fig. 1). Their binding and docking studies implicate a hydrophobic cluster within a SPRY1 loop as a major FKBP binding determinant.

In 2015, 3 studies were published presenting high resolution cryo-EM structures (3.8–6.1 Å) of RyR1.13-15 These studies provide an unprecedented insight into the relationship between RyR structure and function. The structure generated by Marks and colleagues shows that FKBP12 interacts with RyR1 near the clamp domains as described previously by Samso et al. and in a location similar to where Zhang et al. place FKBP12.6 on RyR2.15,61,64 Here FKBP12 interacts with the mutual interface of the bridging solenoid and sits between SPRY1 and 2 (Fig. 1). Given that the N-terminal end of a helix extending from the BrA subdomain also protrudes into this space it is proposed that FKBP12 binding to this site stabilises the SPRY1-SPRY2-BrA interface. The increased rigidity of this region may explain the stability in channel function described by the same group.32,11 Efremov et al. present an almost identical finding for low occupancy endogenously associated FKBP12. Importantl, when comparing the open and closed states of RyR1 the largest changes in conformation occur near to the clamp domains which undergo a 5 Å downward rotation.13 This further suggests that increasing the rigidity of this region is likely to impact channel function. Interestingly, the high resolution cryo-EM studies provided conflicting results for the locations of the SPRY domains 1, 2 and 3. In at least one study this can be attributed to the low regional resolution which led to an ambiguous assignment of the domains.15

When paired with the earlier biochemical data the recent structures strengthen the notion that the interaction of FKBP occurs at multiple sequence sites. They suggest that the N-terminus may contain multiple interacting residues spread through the SPRY domains (amino acids 643–794 and 1073–1205). FKBP’s interaction with α-solenoid 1 supports the work of Chen and colleagues that amino acids 1815–1855 are crucial for FKBP12.6 association with RyR2. However, they question the importance of the central region (2427–2428) in FKBP binding as α-solenoid 2 does not appear to be involved.13

Subsequent to the structures of RyR1, Peng et al. presented the first high resolution structure for RyR2 in 2016.16 As with the structures of RyR1, the regional resolution around the FKBP12.6 binding site was low making detailed assignment of the SPRYs difficult. The low density in this location could suggest a high level of flexibility, particularly in the absence of FKBP association. However, comparison of the sequences with RyR1 (SPRY and Handle domains) suggests that the local structure should be almost identical. Importantly, as with the structures for RyR1 the shift between the open and closed states results in a movement in the periphery of the protein with the SPRY domains rocking downwards and rotating counter-clockwise when observed from the luminal side. This conserved movement and site of FKBP association between RyR isoforms suggests that any cross-linking or stabilization of structures in this region may affect channel gating in both isoforms.

Spatial relationship between FKBP, phosphorylation sites and CPVT mutations

As described above one of the many controversies surrounding FKBP-RyR interaction is the effect of RyR2 phosphorylation and RyR2 mutations causative of CPVT. Marks and colleagues have associated FKBP12.6 dissociation to both channel modifications, whereas others have been unable to replicate their findings. What new insight on this discussion can be obtained now that the location of the FKBP binding site has been identified?

The phosphorylation ‘hot-spot’ domain (tandem repeat 3–4), containing both S2808 and S2814 was originally assigned to domain 10 of RyR, however the new higher resolution structures have shown that S2843 in RyR1 (analogous to S2808 in RyR2) actually forms part of domain 665 (Fig. 2). Interestingly, this region is another poorly resolved element in the protein structure suggesting it is highly flexible.66 This could be due the different conformations induced by phosphorylation. However, studying the structure of the fully dephosphorylated form does not improve resolution suggesting other factors are responsible.15 Notwithstanding, the distance between S2843 and the FKBP binding site is (>60 Å, Fig. 2) suggesting any effect S2843 phosphorylation has on FKBP binding must be via an allosteric mechanism.13-15 The location of the ‘hot-spot’ within the macro-structure is also consistent with earlier lower resolution studies inserting GFP into the site (Fig. 2) further confirming the
distance between the phosphorylation sites and FKBP binding site.\cite{67}

With regard to CPVT mutations, the relatively large distribution of these mutations within the sequence of RyR2 makes it difficult to directly connect FKBP12.6 dissociation to all of them. Indeed when Peng et al. mapped many mutations to the high resolution structure of RyR2 they found that the mutations are distributed throughout the structure. Interestingly, several groups of mutations were found to be located at domain interfaces suggesting that direct changes in inter-domain interactions may underlie the effect of these mutations on channel function.\cite{16} However, as with phosphorylation this does not exclude the possibility that CPVT mutations throughout the structure alter the FKBP binding pocket via allosteric means.

**Concluding remarks**

The sensitivity of FKBP binding to allosteric changes may underlie the conflicting results from different groups as to the exact location of the FKBP binding site within the RyR primary sequence. Moreover, it is likely that there is not a single sequence in the primary structure of RyR that is responsible for FKBP binding. The high level of RyR flexibility particularly around the FKBP binding site may permit distant changes in protein structure to make small changes in the FKBP binding-pocket subtly altering its affinity for the protein. However, the spatial relationship between FKBP and S2808 strongly suggests that there is no direct modulation of FKBP binding due to S2808 phosphorylation. Although unlikely given the majority of the evidence, it however still remains possible that phosphorylation at this site can indirectly affect FKBP binding through an allosteric mechanism. Such a model has previous been proposed by Ikemoto and colleagues,\cite{68} who before the high resolution structures, suggested that mutations can affect RyR folding or inter-domain interactions, which in turn alter FKBP-binding pocket conformation.

Finally, the fact that both FKBP isoforms interact with the same region in RyR2 supports the theory that “competition” between FKBP12 and FKBP12.6 is an important determinant of the final functional result. This is an important consideration for future single channel and recombinant cell models.

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No potential conflicts of interest were disclosed.

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