evidence for protein conformational changes upon ATP or caffeine binding. We observed a similar binding affinity of the expressed RyR2 central domain for both compounds, with an EC50 ~100μM. The presence of the individual mutations made no apparent difference to the EC50. However, there was an increased initial fluorescence value observed for each of the mutant constructs compared to WT. Analysis of the central domain sequence revealed homology with conserved motifs typical of P-loop kinases. Interestingly, many of the CPVT mutations present in this region are coincident with sites corresponding to the Walker A, Walker B and LID motifs in ATP binding proteins.

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Effect of FKBP12 in the Conformational Dynamics of RyR1

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The ryanodine receptor (RyR) is an intracellular ion channel with an important role in depolarization-induced Ca^{2+} release in excitable cells. For the skeletal muscle isoform RyR1, the open and closed conformations were determined at 10 Å resolution by cryoEM, and their structural analysis suggest that RyR1, with four inner helices forming a helical bundle, has a similar gating mechanism to that of K+ channels. Upon gating, RyR1's large cytoplasmic domain also undergoes conformational changes, underlying a long-range communication pathway between the ion gate and effectors bound more than 130 Å away. One of such effectors is the FK506-binding protein of 12 kDa (FKBP12), which we localized at the interface of the ''handle'' and the ''clamp'' domains of RyR1. At the functional level, and under activating [Ca^{2+}] concentrations, single channel experiments with purified RyR1 show that FKBP12 increased RyR1's Po and redistributed its subconductance states towards higher conductance levels. At the structural level, we found that under sub-activating [Ca^{2+}] concentrations and in the absence of FKBP12, RyR1's cytoplasmic domain appears to be a structural intermediate of the well-defined open- and closed-state RyR1-FKBP12 conformations, whereas the inner helices remain in the closed conformation. Overall, our studies suggest that while [Ca^{2+}] exerts a dominant effect in RyR1's gating, FKBP12 changes the energy landscape of RyR1's structural transitions.

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Stabilization of the Skeletal Muscle Ryanodine Receptor Ion Channel-FKBP12 Complex by the 1,4-Benzothiazepine Derivative S107

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Activation of the skeletal muscle ryanodine receptor (RyR1) complex results in the rapid release of Ca^{2+} from the sarcoplasmic reticulum and muscle contraction. Dissociation of the small FK506-binding protein 12 subunit (FKBP12) increases RyR1 activity and leads to the impairment of muscle function. The 1,4-benzothiazepine derivative JTV519, and more specific derivative S107 (2,3,4,5,-tetrahydro-7-methoxy-4-methyl-1,4-benzothiazepine), are thought to improve skeletal muscle function by stabilizing the RyR1-FKBP12 complex. Here, we report that SR vesicles enriched in RyR1 bound a large number of S107 molecules with micromolar affinity. The effects of S107 and FKBP12 on RyR1 were examined under conditions that altered the redox state of RyR1. In SR vesicles, S107 increased FKBP12 binding to RyR1 in presence of reduced glutathione and the NO-donor NOC12. S107 was without effect in the presence of oxidized glutathione. Addition of 0.15 μM FKBP12 to SR vesicles prevented FKBP12 dissociation, whereas in the presence of oxidized glutathione and NOC12 FKBP12 dissociation was observed in skeletal muscle homogenates that contained 0.43 μM myoplasmic FKBP12, and this dissociation was attenuated by S107. In single channel measurements, pretreatment of FKBP12-depleted RyR1s with S107 in the absence and presence of NOC12 augmented the FKBP12-mediated decrease in channel activity. The data suggest that S107 can reverse the harmful effects of redox active species on SR Ca^{2+} release in skeletal muscle.

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Investigating the Relationship between FKBP Structure and the Ability to Activate RyR Channels

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We have recently demonstrated, both at the single-channel level and in isolated cardiac cells (Galfre et al., (2011) *PLoS ONE*, 7, Issue 2, e31956), that the cardiac RyR (RyR2) is activated by FKBP12 and that this effect is regulated by the antagonistic action of FKBP12.6. We now investigate whether FKBPs can also activate skeletal RyR (RyR1) and examined the relationship between FKBP structure and the ability to activate RyR1 and RyR2. We generated a mutant FKBP12 ($FKBP12_{E31Q/D32N/W59F}$) where the amino acids Glu^{31}, Asp^{32} and Trp^{59} were mutated to the respective residues found in FKBP12.6 (Gln, Asn and Phe). The effects of the wild type and mutant $FKBP12_{E31Q/D32N/W59F}$ proteins on the single-channel gating of RyR1 and RyR2 were then compared. Our results show that FKBP12.6 promotes activation of RyR1 whereas FKBP12 promotes activation of RyR2. The FKBP12 mutant, $FKBP12_{E31Q/D32N/W59F}$ did not alter RyR2 Po (n=5) but caused significant activation of RyR1; for example Po was 0.021 ± 0.008 before and 0.110 ± 0.026 after addition of cytosolic 1 μM $FKBP12_{E31Q/D32N/W59F}$ (SEM; n=7; $^{**}p<0.01$)). The FKBP12 mutant, therefore, no longer behaves like FKBP12 but instead mimics the actions of FKBP12.6 on both RyR isoforms. In conclusion, we demonstrate that FKBP12 and FKBP12.6 specifically activate RyR2 and RyR1, respectively. Our results also suggest that the ability of FKBP12 and FKBP12.6 to activate RyR channels is contained within just three amino acid residues of FKBP12 (Glu^{31}, Asp^{32} and Trp^{59}) and the corresponding residues in FKBP12.6 (Gln^{31}, Asn^{32} and Phe^{59}). These results shed new light on the interactions of FKBPs with RyR channels and will be important for understanding the dual role of FKBP12 and FKBP12.6 as regulators of sarcoplasmic reticulum Ca^{2+}-release in cardiac and skeletal muscle.

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FRET-based Mapping of Regions involved in FKBP12.6 Binding to the Type 1 Ryanodine Receptor

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The type 1 ryanodine receptor (RyR1) is an intracellular calcium channel that plays an integral role in skeletal muscle excitation-contraction coupling. The functional properties of this protein are regulated by numerous associated proteins, including FKBP12, a skeletal muscle protein that stabilizes full RyR1 channel gating. While the binding site of FKBP12 was originally identified at position 2458 of RyR1, subsequent reports have localized additional binding determinants within the N-terminal domain of RyR1. In this study, we used fluorescence resonance energy transfer (FRET) to determine the orientation and proximity of FKBP relative to sites either within the N-terminal domain or near amino acid position 2458. We utilized the cardiac muscle isoform FKBP12.6 (which binds to RyR1 at the same site as FKBP12 but with higher affinity and stability) labeled with the FRET donor Alexa Fluor 488 at either position 14, 44, 49, or 85. Specific binding of each labeled FKBP to RyR was confirmed in permeabilized HEK-293T cells expressing wtRyR1. Weak energy transfer was observed from FKBP12.6 to the FRET acceptor Cy3NTA, which was targeted to poly-histidine ''tags'' placed in the N-terminal domain. This result suggested that the acceptors were not proximal (~90Å away) to FKBP12.6 bound to RyR1. Next, we measured FRET from FKBP12.6 to Cy3NTA targeted to positions 2157, 2341, 2502 and 2777 of RyR1. FKBP12.6 binding to constructs containing His-tags at positions 2157 and 2502 was significantly reduced relative to binding to wtRyR1. In contrast, we detected strong and weak FRET efficiencies from FKBP12.6 to positions 2341 and 2777, respectively. These data suggest that FKBP12.6 binds near amino acid position 2341 but far from the N-terminal domain of RyR1. Supported by NIH grant R01AR059124 (to JDF and TG) and R01HL092097 (to RLC).

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Physiological Role(S) of RyR1 in Smooth Muscle Cells

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Type 1 ryanodine receptor (RyR1) is a key protein involved in the regulation of the intracellular Ca^{2+} concentration in skeletal muscle cells, playing a crucial role in muscle contraction by releasing Ca^{2+} from the sarcoplasmic reticulum after plasma membrane depolarization. Dysregulation of calcium signals due to defects in RyR1 have been associated with a wide range of primary neuromuscular disorders, including Malignant Hyperthermia Susceptibility and a number of congenital myopathies including Core Myopathies, some forms of Centronuclear myopathy and congenital fiber type disproportion. Though RyR1 is preferentially expressed in skeletal muscles recent data has shown that it is also expressed in some areas of the central nervous system, in some cells of the immune system (B-lymphocytes and dendritic cells) and in smooth muscle cells. Thus mutations in *RYR1* (the gene encoding RyR1) may lead to alterations of Ca^{2+} homeostasis not only in skeletal muscle, but also in other tissues expressing this intracellular calcium release channel.