Further consideration of FKBP12 association with RyR1 and RyR2 and regulation of RyR channel activity

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Ryanodine receptor (RyR) ion channels release the Ca^{2+} required for contraction in striated muscle and are essential for heart beat and movement and consequently are essential for life. The opening of the channels at rest and during excitation-contraction coupling is regulated by many factors including the 12.0 and 12.6 FK506 binding proteins, FKBP12 and FKBP12.6, which are closely associated with the ion channels. FKBP12 alone is expressed in skeletal muscle, while both isoforms are expressed in the heart in a highly species-specific ratio. FKBP dissociation from RyRs is implicated in skeletal and cardiac myopathies, including potentially fatal arrhythmia. Yet at a molecular level their regulation of the ion channel is poorly understood and divergent effects of FKBP removal and re-association with isolated RyRs have been reported. It is generally agreed that dissociation of the FKBP12 from RyR1 leads to increased channel activity and the appearance of sub-maximal conductance (sub-state) openings. On the other hand, stripping the FKBPs from RyR2 channels has been reported to either activate or to have no effect on RyR2 activity. Adding exogenous FKBPs back to RyR1 and RyR2, stripped of (or lacking) endogenous FKBPs, has been variously reported to activate, inhibit or have no effect on channel activity. Curiously, although biochemical studies indicate that FKBP12.6 binds to RyR2 with a Kd of ~0.7 nM, and FKBP12 with a Kd of ~200 nM, when exogenous FKBPs are added to FKBP-depleted RyRs, concentrations as high as 10 micromolar have been used to reverse the effects FKBP depletion. Such high concentrations suggest a low affinity interaction, which contradicts the high affinity Kd measurements and the long held concept that FKBP12 binds "tightly" to RyR channels.

To further explore the effects of FKBP12 on RyR, we have systematically examined the amounts of FKBP associated with RyR1 and RyR2 at different stages of RyR isolation and purification using co-immunoprecipitation and co-sedimentation. The effect of adding FKBP12 to native RyRs (in SR vesicles) and purified RyR1 and RyR2 channels has examined and the concentration-dependence of the effect surveyed. RyR1 was obtained from rabbit skeletal muscle and RyR2 from sheep heart. Native or purified channels were incorporated into artificial lipid bilayers and single channel currents recorded using standard techniques. Human FKBP12, expressed as a GST-fusion protein expressed in *E.coli* in a pHUE vector with a GST tag, purified by glutathione-agarose affinity chromatography, and used for experiments, or the GST cleaved and FKBP12 purified. Either GST-FKBP12 or purified FKBP12 was added to the cytoplasmic solution in bilayer experiment. Results: **1)**. Amounts of FKBP12 associated with RyR1, or FKBP12 and 12.6 associated with RyR2, steadily declined during processing from the cell homogenate through to the purified channel. **2)**. A small amount of endogenous FKBP remained associated with purified channels, and was reduced to undetectable levels by incubation with rapamycin. **3)**. Adding excess exogenous GST-FKBP12 to native RyR channels resulted in a twofold increase in total FKBP bound to the channels, with a significant fraction of exogenous FKBP exchange with endogenous FKBP. **4).** The following results from channel studies, illustrate the complexity of the functional coupling (important trends in bold). --

1. FKBP12 1 pM-1 nM:
   - Purified RyR2: **increase in 6 of 12 channels**; decrease, 2 of 12; no change, 4 of 12.
   - Native RyR2: increase in 0 of 15 channels; decrease, 2 of 15; **no change, 13 of 15**.
   - Native RyR1: increase in 1 of 20 channels; decrease, 5 of 20; **no change, 14 of 20**.
2. FKBP12 5 nM-10 nM:
   - Purified RyR2: **increase in 9 of 16 channels**; decrease, 3 of 16; secondary decrease, 3 of 16; no change, 4 of 16
   - Native RyR2: increase in 3 of 16 channels; decrease, 3 of 16; **no change, 10 of 16**.
   - Purified RyR1: **increase in 7 of 10 channels**: slow decrease, 2 of 10; no change, 1 of 10.
   - Native RyR1: increase in 3 of 14 channels; decrease, 1 of 14; **no change, 10 of 14**.
3. FKBP12 200 nM:
   - Purified RyR2: **increase in 5 of 5 channels**; **secondary decrease, 3 of 5**; **further decline with S107, 2 of 2.**
   - Purified RyR1: **increase in 6 of 6 channels**; **secondary decrease, 2 of 6**; **further decline with S107, 3 of 3.**

In addition: sub-state activity was consistently reduced and full openings stabilised only with 200 nM FKBP12. S107 which stabilises FKBP binding to RyRs, added alone had no effect on channel activity. There was no difference between actions of GST-FKBP12 and purified FKBP12.

The ease of dissociation of FKBP12 from RyRs during processing suggests low affinity binding, while FKBP12 remaining associated with purified RyRs indicates high affinity binding. Strong activation with 200 nM

FKBP12, with longer exposures inducing inhibition that was accelerated by S107, indicating a high affinity activation and low affinity inhibition. This suggest multiple binding sites for FKBP12 on the RyRs with different binding affinities. Our current hypothesis, assuming 4 identical FKBP12 binding sites per tetramer, is that FKBP12 binds to one site with high affinity and to the other 3 sites with decreasing affinity (with a negative co-operativity). Low affinity binding inhibits channels, while higher affinity binding activates channels.