FKBP12.6 activates RyR1: investigating the amino acid residues critical for channel modulation

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SUPPORTING MATERIAL

MATERIAL AND METHODS

Preparation of mutant FKBPs

Briefly, a double mutant ($FKBP12_{E31Q/D32N}$) was first generated using the human FKBP12 gene previously subcloned in pET-28a as a DNA template (15). A plasmid containing the $FKBP12_{E31Q/D32N}$ gene was synthesized by PCR in the presence of complementary synthetic oligonucleotide primers containing the desired mutations. The resulting PCR product was digested with the restriction endonuclease DpnI (which cleaves only when its recognition site is methylated) to remove all template DNA. This was then used to transform-XL1-Blue competent cells (Stratagene-Agilent Technologies, UK). The plasmids were then isolated from the transformants and purified using the QIAprep Spin Miniprep kit (Qiagen, USA). To verify the identity of the mutants, the full length DNA was sequenced by Gene Service, University of Oxford. This plasmid was subsequently used as a DNA template to make FKBP12 $_{E31Q/D32N/W59F}$ triple mutant and the same procedure described above was then followed. The protein $FKBP12_{E31Q/D32N/W59F}$ was expressed in *Escherichia coli* BL21 (DE3) Codon Plus competent cells and purified by fast protein liquid chromatography (FPLC) as previously described for the wild-type FKBP12 (15).

Detection of FKBP proteins in skeletal heavy SR

Control heavy SR (HSR) membrane vesicles and rapamycin pre-treated HSR vesicles (as described in the main methods) were separated on a 15% SDS-PAGE and subsequently stained with Coomassie brilliant blue (CBB). Gel slices corresponding to the molecular weight of FKBP protein (12 kDa) were excised and subjected to in-gel tryptic digestion using a ProGest automated digestion unit (Digilab UK). The peptides were subsequently fractionated using a Dionex Ultimate 3000 nanoHPLC system and analysed using tandem mass spectrometry as previously described (15). Data acquisition was carried out using Xcalibur v2.1 software (Thermo Fisher Scientific, Leicestershire, UK). The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Fisher Scientific, Leicestershire, UK) with searches performed against the UniProt rabbit database by using the SEQUEST algorithm with the following criteria; peptide tolerance = 10 ppm,

trypsin as the enzyme, carboxyamidomethylation of cysteine as a fixed modification and the oxidation of methionine as a variable modification. The reverse database search option was enabled and all data was filtered to satisfy false discovery rate (FDR) of less than 1%.

Lifetime analysis

Po and lifetime distributions were calculated from 3 min of continuous recording using TACfit 4.2.0 software (Bruxton Corporation, Seattle, USA). Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 ms in duration were not fully resolved and were excluded from lifetime analysis. Individual lifetimes were fitted to a probability density function (pdf) by the method of maximum likelihood (65) according to the equation:

$$g(x) = \sum_{i=1}^{N} a_i g_o(x - \ln \tau_i)$$

Where $\ln\tau_i$ is the logarithm of the *i*th time constant and a_i is the fraction of the total events represented by that component (65).

RESULTS

Effects of FKBP12 and FKBP12.6 on the gating of RyR2

Fig. S1 shows typical examples of how FKBP12 and FKBP12.6 affect RyR2 gating. In A, 1 pM FKBP12 increases Po demonstrating the high affinity of the interaction. In B, even concentrations of FKBP12.6 above those thought to be relevant physiologically have no more than a slight tendency to increase Po and no inhibition is observed.

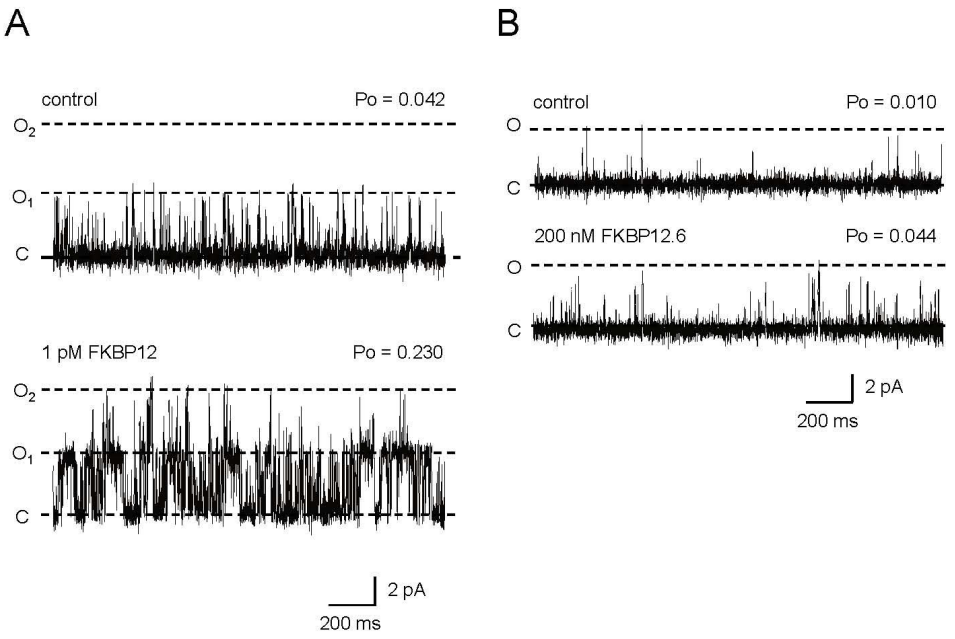

Fig. S1. Sheep cardiac RyR2 is effectively activated by FKBP12 but not by FKBP12.6

(*A*) A typical single-channel experiment showing control recordings in the presence of 10 μM cytosolic Ca^{2+} alone (top trace) and marked activation of RyR2 after addition of 1 pM FKBP12 (bottom trace). (*B*) A separate experiment showing the typical effects of FKBP12.6. The top trace shows the gating behaviour of the channel in the presence of 10 μM cytosolic Ca^{2+} alone and the bottom trace shows the effects of adding cytosolic FKBP12.6. The Po values are indicated above the traces. Dashed lines indicate open (O_1, O_2) and closed (C) channel levels, respectively.

Lifetime analysis

Fig. S2 shows lifetime analysis for a typical single RyR1 channel gating before and after treatment with FKBP12.6 illustrating that the open lifetime distribution is not altered (except for an increase in event number) by FKBP12.6, even at high concentrations. The closed lifetime distribution is characterised by an increased proportion of brief closings demonstrating that Po is increased because of an increase in opening frequency.

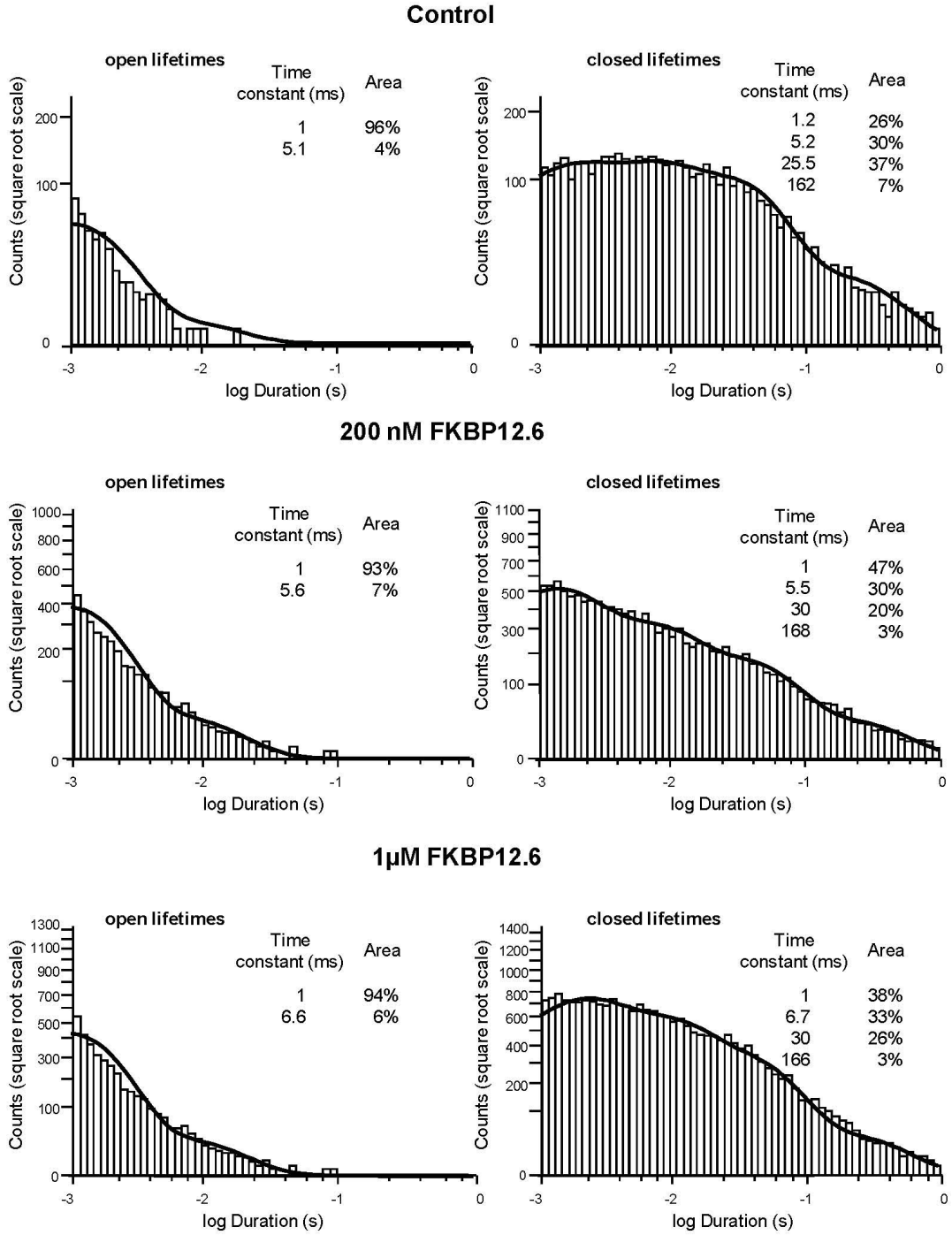

Fig. S2. The effects of FKBP12.6 on open and closed lifetime distributions of rabbit skeletal RyR1

Open (left) and closed (right) lifetime distributions and pdfs of a typical RyR1 channel in the presence of 10 µM cytosolic Ca^{2+} alone (control) and after sequential addition of 200 nM and 1µM FKBP12.6. The best fits to the data were obtained by maximum likelihood fitting (see Methods). Time constants and percentage areas are shown for each distribution.

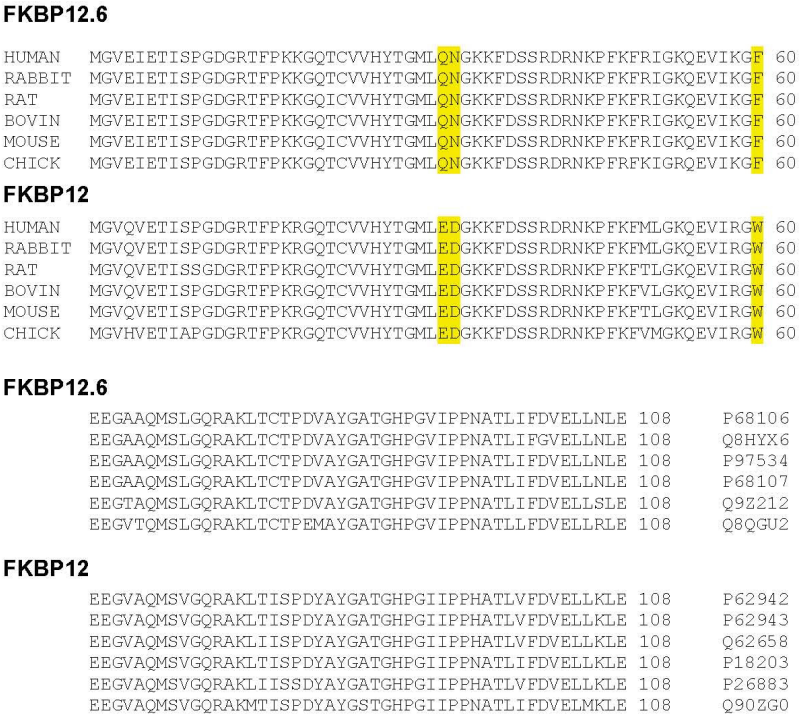

Fig. S3. Multiple sequence alignment of FKBP12 and FKBP12.6
The amino acid sequences of FKBP12.6 and FKBP12 from human, rabbit, rat, cow, mouse and chicken are compared. The corresponding UniProtKB entries are shown at the end of each sequence. The three conserved residues which were mutated in the protein FKBP12 $_{E31Q/D32N/W59F}$ are highlighted in yellow.

Mass spectrometric analysis

In order to verify whether rapamycin is able to completely strip FKBPs from skeletal HSR membranes, mass spectrometric analysis was performed on rapamycin pre-treated HSR vesicles. Untreated HSR membranes were analysed as control. In both samples, FKBP12 was identified with high confidence (FDR< 1%) indicating that rapamycin treatment is unable to completely displace FKBP12 from RyR1 channels. The mass spectrometry results for FKBP12 in HSR and rapamycin pre-treated HSR samples are compared in Table 1.

		HSR			Rapamycin pre-treated HSR		
Accession	**Description**	**Coverage**	**Total peptides**	**Unique Peptides**	**Coverage**	**Total peptides**	**Unique Peptides**
P62943	FKBP12	76.59	75	9	29.63	17	4

Table 1. Mass spectrometry data for FKBP12 in HSR and rapamycin pre-treated HSR samples. The table compares the FKBP12 protein peptides that were recovered including those that were identified with high confidence (<1% FDR) in HSR and rapamycin pre-treated HSR samples. Coverage indicates the percentage of the protein sequence covered by identified peptides. Total peptides represents the total number of identified peptide sequences (peptide spectrum matches) for the protein, including those redundantly identified. Unique peptides are the numbers of unique peptide sequences detected.

Rapamycin treatment

FKBPs are dissociated from sheep cardiac HSR by incubation in 400 mM KCl during the sucrose gradient step (15) and therefore the RyR2 channels incorporated into bilayers after fusion of SR vesicles will already be depleted of FKBPs. Hence, any effect of rapamycin on RyR2 channel function is not likely to be related to dissociation of FKBPs from the channel but to a direct effect of rapamycin on the channel. Addition of rapamycin (20 μM) to the cytosolic side of RyR2 channels incorporated into bilayers (Fig. S4) caused huge increases in RyR2 activity even though FKBPs had already been dissociated by the 400 mM KCl sucrose gradient step (Po was 0.051±0.007 before and 0.564±0.072 (SE; n=5; $**p<0.01$) after treatment). This increase in Po was not reversed by perfusing away the rapamycin from the cytosolic chamber. Addition of FKBP12.6 could not reverse the effects of rapamycin although subsequently lowering the free [Ca^{2+}] to sub-activating levels completely shut the channels.

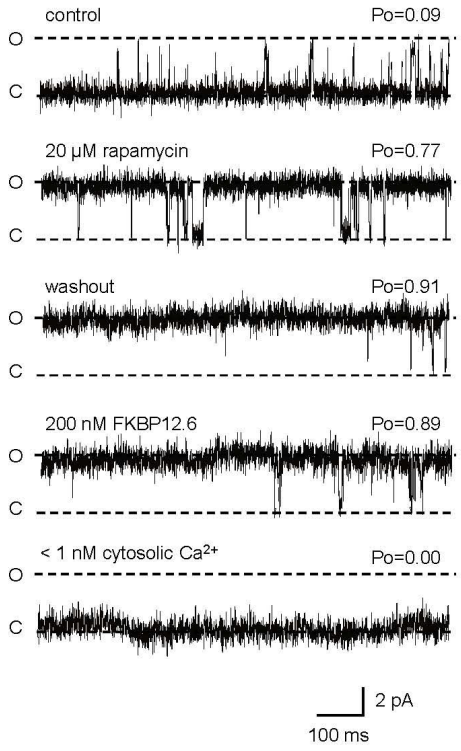

Fig. S4. The effects of rapamycin on sheep cardiac RyR2 channel behaviour

The top trace shows a typical experiment illustrating RyR2 channels gating in the bilayer and activated by 10 μM cytosolic Ca^{2+} alone. Subsequent addition of cytosolic rapamycin (20 μM) leads to a marked increase in channel Po. Cytosolic wash-out of rapamycin shows that the effects of rapamycin are irreversible. Subsequent addition of cytosolic FKBP12.6 (200 nM) has no effect. The bottom trace shows that RyR2 is still regulatable by cytosolic Ca^{2+} since lowering the free [Ca^{2+}] to sub-activating levels (<1 nM) by addition of 10 mM EGTA, completely closes the channel. The Po values are shown above the relevant trace. O and C represent the open and closed channel levels respectively.