FKBP12.6 binding of ryanodine receptors carrying mutations associated with arrhythmogenic cardiac disease

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In the present paper we show that distinct human RyR2 (ryanodine receptor type 2) inherited mutations expressed in mammalian cells exhibit either unaltered or increased FKBP12.6 (12.6 kDa FK506-binding protein) binding compared with the wild-type. Oxidizing conditions result in decreased FKBP12.6 binding, but to the same extent as for the wild-type. Our findings suggest that FKBP12.6 regulation of RyR2 is unlikely to be the primary defect in inherited arrhythmogenic cardiac disease.

Key words: arrhythmogenic cardiac disease, FKBP12.6, immunophilin, protein interaction, redox regulation, ryanodine receptor.

INTRODUCTION

CPVT (catecholaminergic polymorphic ventricular tachycardia) is an inherited arrhythmogenic disease characterized by adrenergically mediated bidirectional or polymorphic ventricular tachycardia, leading to syncope and/or sudden cardiac death [1,2]. CPVT is a highly malignant disease (mortality rates of 30–35%) with incomplete penetrance, manifesting in childhood and adolescence. Patients with CPVT have structurally normal hearts and typically present with ventricular arrhythmias due to physical or emotional stress. ARVD2 (arrhythmogenic right-ventricular dysplasia type 2) is a related disease with mild structural abnormalities of the right-ventricular myocardium in addition to stress-induced polymorphic ventricular tachycardia. CPVT and ARVD2 have been linked with mutations in RyR2 (ryanodine receptor type 2), the intracellular channel mediating rapid Ca²⁺ release from the SR (sarcoplasmic reticulum) that triggers cardiac muscle contraction [3].

RyR2 forms a complex with a small accessory protein, FKBP12.6 (12.6 kDa FK506-binding protein), a member of the immunophilin family that stabilizes channel conductance and facilitates channel closure [3]. Defective regulation of RyR2–FKBP12.6 association has been suggested to play a role in the pathogenesis of acquired and inherited cardiac disease. Marks and colleagues [4–7] proposed that PKA (protein kinase A)-mediated ‘hyper-phosphorylation’ of RyR2, resulting in dissociation of FKBP12.6 association has been suggested to play a role in the pathogenesis of acquired and inherited cardiac disease. Marks and colleagues [4–7] proposed that PKA (protein kinase A)-mediated ‘hyper-phosphorylation’ of RyR2, resulting in dissociation of FKBP12.6, underlies the abnormal diastolic SR Ca²⁺ release during β-adrenergic stimulation. This proposal was based on their observation that mutant RyR2 channels had reduced affinity for FKBP12.6 and that mice haploinsufficient for FKBP12.6 displayed exercise-induced ventricular arrhythmias that were prevented by β-adrenergic blockers or JTV519, a drug that restored the RyR2–FKBP12.6 association. However, these results are not consistent with those obtained in other laboratories [8–12].

The RyR2 ‘hyper-phosphorylation’ hypothesis, which describes an ensuing loss of FKBP12.6, is an attractive mechanism that predicts an increased diastolic Ca²⁺ release leading to delayed after-depolarization, the underlying cause of cardiac arrhythmias in heart failure and sudden cardiac death. However, FKBP12.6 dissociation from RyR2 may also result from a different causal mechanism. For example, we have recently demonstrated that oxidized RyR2 has a decreased affinity for FKBP12.6 [13]. Thus, in the present study, we have assessed the interaction of FKBP12.6 with recombinant wild-type and mutant (R176Q, S2246L and R4497C) RyR2 channels under reducing and oxidizing conditions, to determine whether a redox mechanism may play a role in RyR2 disease pathogenesis. The mutation R176Q was initially associated with ARVD2 [14], whereas S2246L and R4497C were causative of CPVT [15]. All three mutations have been previously characterized in in vitro systems, and channel dysfunction has been correlated with both FKBP12.6-dependent and -independent mechanisms [4,8,9,16].

MATERIALS AND METHODS

Cell-culture reagents were from Invitrogen. The in vitro protein expression system (TNT® T7 Quick Coupled Transcription/Translation System) was from Promega. Radioactive sulphur (Pro-Mix; GE Healthcare) contained ~70% L-[³⁵S]methionine and 30% L-[¹⁴C]lysine. Electrophoresis reagents were from Bio-Rad. CHAPS and rapamycin were from Calbiochem, whereas all other reagents were from Sigma. Redox reagents were prepared as 100× stock solutions, divided into portions and stored frozen at −80°C. H₂O₂ was always added fresh from an aq. 30% (v/v) solution at 4°C. Cell-free protein expression, SDS/PAGE, autoradiography, Western-blot and densitometry analysis were carried out as described previously [13]. Statistical analysis of densitometric data was performed using an unpaired Student’s t test.

Plasmid construction

Preparation of plasmids expressing EGFP (enhanced green fluorescent protein)-tagged human RyR2 mutants R176Q, S2246L and R4497C has been described previously [8,16]. Untagged

Abbreviations used: ARVD2, arrhythmogenic right-ventricular dysplasia type 2; CMV, cytomegalovirus; co-IP, co-immunoprecipitation; CPVT, catecholaminergic polymorphic ventricular tachycardia; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; FKBP12.6, 12.6 kDa FK506-binding protein; HEK-293, human embryonic kidney-293; PKA, protein kinase A; RyR2, ryanodine receptor type 2; SR, sarcoplasmic reticulum.

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versions were prepared by replacing the MluI–SpeI ~1.3 kb fragment [containing part of the CMV (cytomegalovirus) promoter and the entire EGFP sequence] with a PCR-generated MluI–SpeI ~0.6 kb fragment recreating the CMV promoter, but without EGFP, using the following oligonucleotide primers:

**Forward:** GCACCGGTGTTCTAGGCCATATATGGAG

**Reverse:** GCCGACTA GTAGCGCTA CGGATCTGAC

Untagged channels were produced in order to avoid potential redox-related problems with the EGFP moiety.

**Cell culture, transfection and microsome preparation**

HEK-293 (human embryonic kidney-293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) foetal bovine serum and 2 mM glutamine in a humidified atmosphere of 5% CO₂ at 37°C. Cells at ~70% confluence were transiently transfected with 24 μg of RyR2 plasmid DNA per 100-mm-diameter Petri dish using the calcium phosphate precipitation method. Cells were harvested 24 h post-transfection, and crude microsomes were prepared as described below. To obtain similar protein levels for RyR2 wild-type and mutants, equal numbers of cells were simultaneously transfected and processed for all four RyR2 constructs.

Crude microsomes were prepared as follows: HEK-293 cells were resuspended in homogenization buffer, then stored at −80°C. Graded amounts of microsomes were processed for the other constructs to obtain uniform total RyR2 content. There was equivalent loading with uniform total RyR2 content. There was equivalent antibody (16B4; Calbiochem) was used in immunoblots that were recovered in the immunoprecipitate (Figure 1C). To compare whether channel activity affects the RyR2–FKBP12.6 interaction, the mutant RyR2 isoform-specific antibody Ab1093 was added at 1:40 dilution and the sample was incubated for 2 h at 4°C, followed by the addition of 20 μl of Protein G Dynabeads (Dynal) and incubation for a further 2 h with continuous mixing. Protein immunocomplexes were isolated with the use of a magnetic-particle concentrator (MPC-S; Dynal) and beads were washed three times with IP buffer. Immunoprecipitated proteins were resuspended in SDS/PAGE loading buffer [60 mM Tris/2% (v/v) SDS, 10% (v/v) glycerol, 5 mM EDTA, 2% (v/v) 2-mercaptoethanol and 0.01% Bromophenol Blue, pH 6.8], heated at 85°C for 5 min, then subjected to SDS/PAGE and autoradiography.

**RESULTS**

We initially determined the relative FKBP12.6 binding affinity of recombinant wild-type and mutant (R176Q, S2246L and R4497C) RyR2 by co-IP assays using solubilized HEK-293 microsomes (normalized for RyR2 and total protein content as detailed in the Materials and methods section) and incubated with [35S]FKBP12.6. Parallel negative controls were processed in the presence of 20 μM rapamycin, a specific inhibitor of RyR2–FKBP12.6 interaction. RyR2 was immunoprecipitated with the RyR2-isoform-specific antibody Ab1093, and the presence of co-precipitated [35S]FKBP12.6 was analysed by SDS/PAGE and autoradiography. As Figure 1(A) shows, all RyR2 constructs displayed rapamycin-sensitive FKBP12.6 binding. Densitometry analysis and normalization with wild-type RyR2 revealed that the R176Q mutant bound ~40% more FKBP12.6 than did the wild-type receptor under identical conditions (Figure 1D; Table 1). The mutant S2246L also displayed ~20% higher binding, whereas the FKBP12.6 interaction of R4497C and wild-type RyR2 were similar. Western-blot analysis indicated that equal amounts of wild-type and mutant RyR2 channels were recovered in the immunoprecipitate (Figure 1C). To compare whether channel activity affects the RyR2–FKBP12.6 interaction, we also conducted co-IP experiments in the presence of 2 mM MgCl₂, a condition that should result in channels being in their closed state (Figure 1B). No effect was apparent, as the results with MgCl₂ were similar to those obtained under ambient conditions (Figure 1E; Table 1).

To address the redox-sensitivity of the RyR2–FKBP12.6 interaction, we treated the solubilized HEK-293 microsomes with a redox reagent (2 mM DTT, 1 mM H₂O₂ or 200 μM diamide) for 30 min at room temperature, followed by incubation with [35S]FKBP12.6. RyR2 was immunoprecipitated with Ab1093 as described above and [35S]FKBP12.6 levels were quantified by autoradiography. Representative autoradiograms are shown in Figures 2(A) and 2(B) and cumulative data, where results are normalized for the control condition (no redox treatment), are presented in Figures 2(E) and Table 2. The thiol-group-oxidizing agents H₂O₂ and diamide decreased FKBP12.6 binding to wild-type RyR2 by ~10% and ~50% respectively. This is consistent with results obtained for native RyR2 from pig heart [13], although the H₂O₂ effect is less pronounced for the recombinant protein. However, in contrast with the native protein, where there was largely no effect, the reducing agent DTT decreased FKBP12.6 binding to recombinant RyR2 by ~20% compared with the control.
Immunophilin binding of RyR2 mutants linked to cardiac sudden death

Figure 1 Interaction of recombinant RyR2 channels with FKBP12.6

Solubilized HEK-293 microsomes expressing wild-type (WT) or mutant (R176Q, S2246L or R4497C) RyR2 channels (normalized for RyR2 and total protein content as detailed in the Materials and methods section) were incubated with in-vitro-synthesized haemoglobin-free [35S]FKBP12.6 in the presence (+) or absence (−) of 20 μM rapamycin, either under ambient conditions (A) or in the presence of 2 mM MgCl₂ (B). RyR2 was immunoprecipitated with Ab1093 and the presence of co-precipitated [35S]FKBP12.6 was analysed by SDS/15%-(w/v)-PAGE and autoradiography. An aliquot of the TNT reaction, 1% of the volume processed in co-IP assays, was included in the autoradiograph to serve as a molecular-mass standard (TNT). (C) Western-blot analysis (SDS/4%-PAGE) using RyR2 isoform-specific Ab 1093 of immunoprecipitated wild-type and mutant RyR2 channels. (D and E) Quantitative densitometry analysis of co-immunoprecipitated [ 35S]FKBP12.6 under ambient conditions (n = 4) (D) or in the presence of 2 mM MgCl₂ (n = 4) (E) with normalization against wild-type RyR2. Results are means ± S.E.M., *P < 0.001, ‡P < 0.05. RAP, rapamycin.

Table 1 Interaction of recombinant RyR2 channels with FKBP12.6

Co-immunoprecipitation experiments to determine the FKBP12.6 binding affinity of recombinant wild-type and mutant RyR2 channels were carried out as described in the legend to Figure 1, followed by quantification of the [35S]FKBP12.6 band by densitometric analysis of autoradiographs and normalization against wild-type (WT) RyR2. The non-specific rapamycin-resistant binding was subtracted from the total to obtain specific FKBP12.6 binding.

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>R176Q</th>
<th>S2246L</th>
<th>R4497C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient (n = 4)</td>
<td>100</td>
<td>140.4 ± 10.6</td>
<td>124.6 ± 6.5</td>
<td>99.4 ± 2.6</td>
</tr>
<tr>
<td>2 mM MgCl₂ (n = 4)</td>
<td>100</td>
<td>149.8 ± 10.2</td>
<td>121.1 ± 4.4</td>
<td>104.3 ± 7.6</td>
</tr>
</tbody>
</table>

The effect of H₂O₂ and diamide on FKBP12.6 interaction with the three RyR2 mutants (R176Q, S2246L and R4497C) was similar to that shown by the wild-type, i.e. they decreased FKBP12.6 binding to the same extent as for wild-type (Figure 2E and Table 2). These results suggest that the point mutations studied here do not alter the diminished FKBP12.6 binding affinity of oxidized RyR2. We also examined whether the closed-state recombinant RyR2 affects the redox sensitivity of the FKBP12.6 interaction, since it was previously found that H₂O₂ has a less pronounced effect on the native channel in its closed configuration [13]. Thus we carried out co-IP assays in the presence of 2 mM MgCl₂, to promote a closed RyR2 channel (Figures 2C and 2D). The results, summarized in Figures 2(F) and Table 2, are similar to those obtained under ambient conditions, indicating the absence of a significant effect of channel activity on the interaction with FKBP12.6.

DISCUSSION

RyR2 mutations predispose affected individuals to cardiac arrhythmias, but present a malignant phenotype only following exercise or emotional stress, indicating the requirement for an additional precipitating factor [1,2]. RyR2-associated cardiac disease is also characterized by incomplete penetrance, since some family gene carriers lead a normal life, apparently able to tolerate the elusive precipitating factor. This ‘trigger’ factor could be reactive oxygen species produced in muscles during exercise [17], suggesting that unaffected individuals may possess an enhanced antioxidant defence system. Notably, increased catecholamine levels following sympathetic-nervous-system activation can lead to cardiomyopathy through the production of reactive oxygen species due to catecholamine autoxidation [18,19].

In the present study we tested the hypothesis that RyR2 mutants have an increased sensitivity to oxidative conditions compared...
Figure 2  Redox-sensitive interaction of recombinant RyR2 channels with FKBP12.6

Solubilized HEK-293 microsomes expressing wild-type (WT) or mutant (R176Q, S2246L, R4497C) RyR2 channels were treated with a redox reagent (2 mM DTT, 1 mM H2O2 or 200 μM diamide) for 30 min at room temperature, followed by incubation with in-vitro-synthesised haemoglobin-free [35S]FKBP12.6, either under ambient conditions (A, B) or in the presence of 2 mM MgCl2 (C, D). RyR2 was immunoprecipitated with Ab 1093 and the presence of co-precipitated [35S]FKBP12.6 was analysed by SDS/15%- (w/v)-PAGE and autoradiography. An aliquot of the TNT reaction, 1 % of the volume processed in co-IP assays, was included in the autoradiograph to serve as a molecular-mass standard (TNT). Quantitative densitometry analysis of co-immunoprecipitated [35S]FKBP12.6 with the wild-type that leads to a more pronounced dissociation of the RyR2-stabilizing FKBP12.6 subunit. This could be either due to decreased FKBP12.6 affinity of RyR2 mutants even at rest, or due to an oxidation-induced decrease in the affinity for FKBP12.6.
We chose to study three mutants, R176Q, S2246L and R4497C, representing one from each of the three mutational loci, because they are the most studied mutants and knock-in animal models are available [1–3]. The recombinant channels were expressed in HEK-293 cells, a cell line that has undetectable levels of both RyR [16,20,21] and FKBP12.6 proteins [12,22,23].

Our results suggest that the impact of the RyR2 point mutations on the interaction with FKBP12.6 is domain- and/or residue-specific. Whereas the RyR2 C-terminally located R4497C mutation did not affect FKBP12.6 affinity, the N-terminal R176Q and central-domain S2246L mutants displayed enhanced binding to FKBP12.6 by ~40% and ~20% respectively (Figures 1D and 1E, and Table 1). The latter result is unexpected, since previous studies have either reported reduced [4,6], or no change [8,9], in FKBP12.6 binding. It has been suggested that RyR2 mutants show diminished affinity for FKBP12.6 only at sub-saturating FKBP12.6 concentrations and no difference on

Table 2  Redox-sensitive interaction of recombinant RyR2 with FKBP12.6

Co-immunoprecipitation experiments to determine the redox-sensitivity of the recombinant RyR2-FKBP12.6 interaction were carried out as described in the legend to Figure 2, followed by quantification of the [35S]FKBP12.6 band by densitometric analysis of autoradiographs and normalization against control (no redox treatment).

(a) Ambient (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DTT</th>
<th>H2O2</th>
<th>Diamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>76.4±2.6</td>
<td>87.5±5.0</td>
<td>55.8±3.2</td>
</tr>
<tr>
<td>R176Q</td>
<td>100</td>
<td>82.0±5.0</td>
<td>91.9±5.3</td>
<td>53.0±5.1</td>
</tr>
<tr>
<td>S2246L</td>
<td>100</td>
<td>77.1±2.8</td>
<td>93.6±2.0</td>
<td>55.6±4.4</td>
</tr>
<tr>
<td>R4497C</td>
<td>100</td>
<td>76.4±2.1</td>
<td>92.7±2.5</td>
<td>57.0±1.4</td>
</tr>
</tbody>
</table>

(b) 2 mM MgCl2 (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DTT</th>
<th>H2O2</th>
<th>Diamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>78.2±2.6</td>
<td>90.7±2.9</td>
<td>52.4±2.1</td>
</tr>
<tr>
<td>R176Q</td>
<td>100</td>
<td>80.7±1.4</td>
<td>87.6±3.8</td>
<td>52.7±2.4</td>
</tr>
<tr>
<td>S2246L</td>
<td>100</td>
<td>80.4±5.0</td>
<td>91.2±4.5</td>
<td>55.3±1.8</td>
</tr>
<tr>
<td>R4497C</td>
<td>100</td>
<td>83.2±2.6</td>
<td>89.8±2.4</td>
<td>58.8±2.5</td>
</tr>
</tbody>
</table>
maximal binding at saturating concentrations [4,6]. Clearly, we have not reached maximal binding in our co-IP experiments, since we find increased rather than unaltered FKBP12.6 binding, at least for two of the three mutants tested. The reasons for the disparate results presumably arise from the differences in experimental conditions. For example, Marks and colleagues [4,6], who reported decreased affinity for FKBP12.6 by RyR2 mutants, assessed binding levels by measuring the amount of exogenous radiolabelled FKBP12.6 co-sedimenting with RyR2-expressing HEK-293 microsomes. However, it should be noted that measurable amounts of FKBP12.6 co-sediment with HEK-293 microsomes non-specifically, as observed with untransfected cells [23]. Further, the above authors also used [3H]ryanodine binding to quantify RyR2 expression levels [4,6], an assay measuring active channels rather than total number. Owing to the higher [3H]ryanodine binding activity of RyR2 mutants, however, this may result in overestimation of the mutant RyR2 expressed in HEK-293 microsomes.

In the present study, FKBP12.6 binding was assessed by co-immunoprecipitating RyR2 specifically from CHAPS-solubilized HEK-293 microsomes pre-incubated with exogenous [35S]FKBP12.6. Importantly, we paid particular attention, through extensive Western blot and densitometric analysis of RyR2-expressing microsomes, to ensure that identical quantities of RyR2 and total microsomal protein were processed in our co-IP assays. Indeed, equal amounts of wild-type and mutant RyR2 channels were recovered in the immunoprecipitated samples (Figure 1C). Interestingly, slightly higher FKBP levels associated with heart SR were reported for the R4497C knock-in mouse, although this could be due to higher levels expressed in the transgenic animal heart [11]. The R176Q knock-in mouse has also been generated, but the RyR2–FKBP12.6 interaction has not been reported [24].

The redox-sensitivity of FKBP12.6 interaction with recombinant wild-type RyR2 expressed in HEK-293 cells was similar to, but not identical with, the native protein from pig heart (Figures 2E and 2F, and Table 2). FKBP12.6 binding decreased by ~50% in diamide-treated RyR2, a result comparable with that obtained for the native protein [13]. The effect of the less potent and more hydrophilic oxidizing reagent H2O2 on recombinant and native RyR2 was to decrease FKBP12.6 binding by ~10% and ~25% respectively [13]. In contrast, the reducing agent DTT diminished FKBP12.6 binding by ~20% for recombinant RyR2, whereas for the native protein it was without effect [13]. It appears that (a) certain accessible cysteine residue(s) that are oxidized in RyR2 expressed in HEK-293 cells can be effectively reduced by DTT and this affects the binding of FKBP12.6. This is likely to be due either to the different redox environment of HEK-293 cells in culture versus cardiomyocytes, or to the presence of accessory proteins in cardiomyocytes that can redox-regulate the aforementioned cysteine residue(s). Our results indicate that the recombinant channel may have a slightly different redox state compared with the native protein, at least with respect to FKBP12.6 binding. Importantly, the redox-sensitivity of the FKBP12.6 interaction with mutant RyR2 channels was very similar to that of the wild-type, irrespective of redox treatment or channel-activation status (Figures 2E and 2F, and Table 2). This suggests that, whether or not RyR2 mutants display increased channel activity due to oxidative conditions compared with wild-type, it is unlikely to involve regulation by FKBP12.6.

In summary, we assessed the FKBP12.6 binding characteristics of recombinant wild-type and mutant RyR2 channels under basal and oxidizing conditions. Our results are not consistent with the hypothesis that CPVT- and ARVD2-linked RyR2 mutations result in decreased affinity for the stabilizing FKBP12.6 accessory protein.

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SUPPLEMENTARY ONLINE DATA
FKBP12.6 binding of ryanodine receptors carrying mutations associated with arrhythmogenic cardiac disease

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Figure S1  Equivalent, low-level phosphorylation of recombinant wild-type and mutant RyR2 channels
Western-blot analysis (SDS/4%-PAGE) of HEK-293 microsomes expressing wild-type (WT) and mutant RyR2 channels using RyR2 isoform-specific Ab1093 (left panel) and anti-phosphoserine antibody (16B4; Calbiochem) (right panel). The ‘198’ on the bottom left-hand side of each panel refers to the position of a protein standard of molecular mass 198 kDa.

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