Ryanodine receptor binding to FKBP12 is modulated by channel activation state

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Summary
Ryanodine receptor (RyR) Ca$^{2+}$ release channels undergo a conformational change between the open and closed states. Its protein modulator, FK506 binding protein 12 (FKBP12), stabilises the channel gating between the four subunits that surround a central Ca$^{2+}$-conducting pore. To understand the interdependence of RyR and FKBP12 binding, physiological and pharmacological agents were used to modulate the RyR open/closed state. ELISA sandwich binding assays showed that FKBP12 binding was dependent on the free Ca$^{2+}$ and was lower at 1-10 $\mu$M free Ca$^{2+}$ compared with 1 mM EGTA and 1 mM Ca$^{2+}$, and this effect was enhanced by the inclusion of 1 mM ATP. Ruthenium red increased the binding of FKBP12. $[^{3}H]$Ryanodine binding confirmed that 1 mM EGTA, 1 mM Ca$^{2+}$ and 1 $\mu$M ruthenium red closed the channel, whereas 1 $\mu$M free Ca$^{2+}$, 1 $\mu$M free Ca$^{2+}$ + 1 mM ATP, or 10 mM caffeine opened it. These binding conditions were used in surface plasmon resonance studies to measure equilibrium binding kinetics. The affinity constant $K_d$ was significantly greater for the closed than the open channel, a change mediated by a decreased dissociation rate constant, $k_d$. The results show that surface plasmon resonance is a powerful technique that can measure differences in RyR1 equilibrium binding kinetics with FKBP12.

Key words: Ryanodine receptor, FKBP12, Surface plasmon resonance, Channel activation state

Introduction
Ryanodine receptors (RyRs) are calcium release channels that function as conduits for Ca$^{2+}$ to pass from the intracellular Ca$^{2+}$ store – the sarcoplasmic reticulum (SR) – into the cytoplasm to regulate muscle contraction, as part of an exquisitely regulated cycle of events termed excitation-contraction (EC) coupling (Fill and Copello, 2002). RyRs must open and close effectively in the interval between each successive depolarisation (Fill, 2003). The RyR is a homotetramer of four (565 kDa) subunits surrounding a central Ca$^{2+}$ pore that act in a co-ordinated way to gate the Ca$^{2+}$ channel (Meissner, 2004). Topology models show that the assembled RyR homotetramer comprises a cytoplasmic domain (foot region) and a transmembrane region, which traverses the SR membrane (Samso and Wagenknecht, 1998; Serysheva et al., 1999). Such studies, comparing the channel in the presence of EGTA with that in Ca$^{2+}$ plus AMP-PCP, have shown a conformational change in the RyR molecule between the respective open and closed states (Orlova et al., 1996; Serysheva et al., 1999).

RyR open probability in bilayers shows a biphasic response to Ca$^{2+}$, being closed at <0.01 $\mu$M Ca$^{2+}$ or 1 mM Ca$^{2+}$, and the greatest open probability being at 1-10 $\mu$M Ca$^{2+}$ (Meissner et al., 1988). ATP enhances the sensitivity to Ca$^{2+}$. The pharmacological modulators, caffeine and ruthenium red, open and close the channel, respectively (Meissner et al., 1988). In situ, RyR1 can be activated by allosteric interaction with the dihydropyridine receptor (DHPR), which acts as a voltage sensor (Numa et al., 1990), or by trigger Ca$^{2+}$ propagated laterally as Ca$^{2+}$ is released via the RyR1 itself – a process termed calcium induced calcium release (CICR) (Fabiato and Fabiato, 1975). In cardiac muscle, CICR is the prime activation mechanism with the trigger Ca$^{2+}$ originating extracellularly through the cardiac DHPR subtype acting as an L-type Ca$^{2+}$ channel (Williams, 1997). Several mechanisms have been proposed for closure of RyRs (Fill, 2003) including inhibitory feedback by mM Ca$^{2+}$ (Wang et al., 2004).

RyR is regulated by accessory proteins, including FK506 binding protein 12 (FKBP12) (Chelu et al., 2004), a member of a protein family characterised by the binding of the immunosuppressant drugs FK506 and rapamycin (Kay, 1996). FKBP12 association with RyR1 was revealed by their persistent co-purification (Collins, 1991; Jayaraman et al., 1992). The RyR/FKBP stoichiometry is one FKBP per RyR subunit (Jayaraman et al., 1992; Timeimer et al., 1995) and topological analysis revealed four FKBP12 molecules bound symmetrically proximal to the N-terminus (Samso and Wagenknecht, 1998). FKBP12 binding modifies RyR function by stabilising subunit interactions, eliminating subconductance states and decreasing the open probability of the channel (Ahern et al., 1997; Brillantes et al., 1994; Chen et al., 1994; Ma et al., 1995). In bilayer studies, RyR1 or RyR2 channels opened and closed together on addition of FKBP12/12.6 – a process termed coupled gating (Marx et al., 1998; Marx et al., 2001).

Dissociation of FKBP12/12.6 from RyR1/RyR2 is proposed as a common mechanism for the dysfunction of both skeletal (Reiken et al., 2003; Ward et al., 2003) and cardiac muscle in heart failure (Wehrens et al., 2004). Point mutations in RyR1
causing central core disease and/or malignant hyperthermia (Dirksen and Avila, 2002) or in RyR2 associated with cardiac sudden death syndromes have been reported (Wehrens and Marks, 2003), and the latter show altered interaction with FKBP12.6. However, the role of RyR/FKBP interaction in both pathologies is disputed (George et al., 2003; Jiang et al., 2002; Stange et al., 2003; Xiao et al., 2005; Xiao et al., 2004). Thus, defining the role of FKBP12/RyR interaction in EC coupling in both normal physiology and pathology, and understanding the mechanism of interaction in normal and mutant RyR, are of fundamental importance.

Here, we have scrutinised the inter-relationship of RyR1 and FKBP12 with respect to the open and closed state of the RyR channel per se. We used surface plasmon resonance (SPR) and recombinant GST-FKBP12 coupled to a Biacore sensor chip. Equilibrium kinetics was measured for native RyR1 with physiological and pharmacological modulators of channel open and closed states. [3H]Ryanodine binding confirmed the open or closed status of the channel in each condition. The equilibrium binding rate and affinity constants segregated dependent on the open and closed state. The major determinant of change was a decreased rate of dissociation of FKBP12 from RyR1 when the channel was closed.

Materials and Methods
Preparation and purification of GST-FKBP12
Partially purified RyR1 was prepared from skeletal muscle microsomes (Blayney et al., 2004). To remove endogenous FKBP12, 1 µM rapamycin was added during solubilisation (Blayney et al., 2004). Western blot was used to detect any remaining endogenous FKBP12 during preparation. Fig. 1A shows that FKBP12 was undetectable in lane B. The calibration using serial dilution of FKBP12 (Sigma) was pre-incubated with RyR1 for 30 minutes. Additions to the binding buffer included 1 mM EGTA, 1 µM free Ca2+ ± 1 mM ATP, 1 µM ruthenium red or 10 mM caffeine. BIA Evaluation software calculated the K_A, k_a and k_d using the base line drift correction algorithm. Experiments were repeated four times using RyR1 preparations from different rabbits.

Results
Removal of endogenous FKBP12
RyR1 was treated with 1 µM rapamycin to remove endogenous FKBP12 during preparation. Fig. 1A shows that FKBP12 was undetectable in lane B. The calibration using serial dilution of GST-FKBP12 (Fig. 1A lanes C-G) showed that it was possible to detect FKBP to a threshold of 26 nM. It was calculated, from the FKBP12/RyR1 stoichiometry of 4:1, that for 250 µg/ml of RyR1 (~10 nM), maximally, 40 nM FKBP12 could be present. None was detected, but subsaturation levels of FKBP12 could remain.

FKBP12 binding to RyR1
ELISA sandwich assays (Blayney et al., 2004) were used to determine if changes in FKBP12 binding to RyR1 could be measured in response to agents (added in the binding buffers) known from the literature to favour either the open or closed state of the RyR channel (Meissner et al., 1988). Fig. 1B shows the effect of free Ca2+ concentration, with data normalised by subtraction of binding at ambient Ca2+ to that in the presence of caffeine. As the free Ca2+ increased, the comparative amount of FKBP12 bound decreased, but the trend was reversed as the Ca2+ concentration rose to 1 mM. The effects were enhanced up to 10 µM Ca2+ by the addition of 1 mM ATP. Comparison of binding at ambient Ca2+ to that in the presence of caffeine or ruthenium red showed that caffeine had no effect, but that FKBP12 binding was increased by ruthenium red (Fig. 1C).

Binding could be specifically inhibited by the inclusion of the inhibitor FK506 (1 µM), being 0.425±0.049 in buffer alone compared with 0.214±0.036 in its presence (P<0.05, n = 5 ± s.e.m., measured as A495 normalised by subtraction of binding to BSA coated to a control well (Blayney et al., 2004); data not shown).
Ryanodine receptor/FKBP12 interaction

Ryanodine binds to the open conformation of RyR and is considered a reliable measure of the open/closed state (Lai et al., 1989). For SPR experiments (see below) 1 mM EGTA, 1 μM free Ca\(^{2+}\) ± 1 mM ATP, 1 mM Ca\(^{2+}\), 10 mM caffeine and 1 μM ruthenium red were the chosen assay conditions based on the results of the ELISA assays (Fig. 1B,C). The results in Fig. 1D, normalised by dividing by the value for 1 mM EGTA, show that binding was 30-60-fold higher in the presence of 1 μM Ca\(^{2+}\) ± 1 mM ATP and 10 mM caffeine than in the presence of 1 mM EGTA, 1 mM Ca\(^{2+}\) or 1 μM ruthenium red compared with the caffeine value, Fig. 1D. Thus, 1 μM Ca\(^{2+}\) ± 1 mM ATP and caffeine opened the RyR1 channel, and it was closed in the presence of 1 mM EGTA and 1 mM Ca\(^{2+}\).

Surface plasmon resonance

Fig. 2A illustrates a typical series of sensorgrams for the
addition of serial RyR1 concentrations using the kinetic inject wizard. Fig. 2B compares the binding of RyR1 to GST-FKBP or GST bound to the chip and shows that there was no binding to the GST moiety. Fig. 2C shows that total binding was incremental with RyR1 protein concentration and was inhibited by 1 μM FK506, a specific inhibitor of FKBP12/protein interactions. Fig. 2D shows that binding to GST-FKBP12 could be inhibited by pre-binding recombinant FKBP12 to the RyR1, confirming that the FKBP moiety of GST-FKBP interacted with the correct binding site on RyR1. The molar ratio of FKBP12:RyR1 in the analyte, expressed as a function of the percentage of maximum binding (without FKBP), showed that 50% inhibition was obtained at a molar ratio ~5:1.

Equilibrium binding experiments were performed using conditions identified by ryanodine binding and ELISA sandwich assays to alter the channel open/closed state and change the amount of FKBP12 bound, respectively. Fig. 3A shows the Langmuir binding equation for a 1:1 binding equilibrium from which the BIA Evaluation software calculated the constants $K_\Lambda$, $k_a$ and $k_d$. Fig. 3B shows the effect of the different binding conditions on $K_\Lambda$, the association equilibrium constant. Those agents that closed the channel (grey columns) showed the greatest affinity between RyR1 and FKBP12, and those agents that opened the channel (white columns) showed a significantly lower value, in comparison with that for 1 mM EGTA. As $K_\Lambda$ is the ratio of $k_a/k_d$ these individual parameters were compared. Fig. 3C shows that the $k_a$ values for open and closed states were broadly similar. By contrast, the dissociation constant ($k_d$) segregated into values with a dissociation rate between $1 \times 10^{-5}$ to $1 \times 10^{-7}$ second$^{-1}$ (closed channel) and $1 \times 10^{-1}$ to $1 \times 10^{-3}$ second$^{-1}$ (open channel) (Fig. 3D). Thus the differences in $K_\Lambda$ (Fig. 3B) could be attributed to a decrease in the rate of dissociation of the closed RyR1 channel from FKBP12.

The dependence of binding on RyR1 analyte concentration

Fig. 3. Equilibrium kinetics of the GST-FKBP and RyR1 interaction. (A) The Langmuir equation for equilibrium kinetics and definition of the constants, where [A] and [B] are the molar concentrations of interactants and [AB] the molar concentration of the product. Constants $K_\Lambda$, $k_a$ and $k_d$ were calculated by BIA Evaluation software for kinetic experiments, as illustrated in Fig. 2. For each data set (B-D), the white and grey bars denote conditions where the RyR1 channel was open or closed, respectively (cf ryanodine binding, Fig. 1). (B) The equilibrium constant, $K_\Lambda$. The data shows that affinity is greatest when the channel is closed. (C) The association constant, $k_a$, which overall shows no substantial variation nor segregation with channel open and closed states. (D) The dissociation constant, $k_d$, which was greatest when the RyR1 channel was open. $n = 4 ± s.e.m., ^*p<0.05$ compared with the EGTA value, Student’s unpaired $t$ test.

Fig. 4. Surface plasmon resonance measurements – proportion of RyR1 binding to GST-FKBP12. RyR1 binding was normalised for GST-FKBP binding to the chip surface in each binding cycle. (A) Relationship between RyR1 bound (RU of RyR1 bound/RU of GST-FKBP12 bound to the chip) and RyR1 added. RyR1 was flowed over the chip in binding buffer containing EGTA (open circles), 1 mM Ca$^{2+}$ (open squares), 1 μM Ca$^{2+}$ (filled diamonds) and 1 μM Ca$^{2+}$ + ATP (filled triangles). (B) The results for 1 μM ruthenium red (open circles) and 10 mM caffeine (filled diamonds). (C,D) Average of data from four experiments, with white and grey columns denoting agents that open or close the channel, respectively. C compares 1 μM Ca$^{2+}$, 1 μM Ca$^{2+}$ + ATP, and 1 mM Ca$^{2+}$ with the value for 1 mM EGTA. D compares the binding with 10 mM caffeine with that for 1 μM ruthenium red. $n = 4 ± s.e.m., ^*p<0.05$, unpaired Student’s $t$ test.
was determined (Fig. 4). Total RU units bound (for each sensorgram) was normalised to the total RU units of GST-FKB12 on the chip and plotted against the RyR protein concentration added. Scatter plots show that binding was linearly related to added RyR1 protein (Fig. 4A,B). The amount bound was greater per unit of added protein for conditions where the channel was closed, compared with open. Fig. 4C,D shows the same data averaged for each experiment and the four individual experiments statistically compared. Fig. 4C shows that for the physiological agents (1 μM Ca^{2+} ± 1 mM ATP) that open the channel, binding was reduced compared with 1 mM EGTA and 1 mM Ca^{2+} (channel closed). In the presence of the pharmacological agent caffeine (channel open), RyR showed significantly less binding to FKB12 than in the presence of ruthenium red (channel closed).

Discussion

FKBP12 is a potent and important regulator of RyR function. It both stabilises the interaction between homotetrameric subunits (Ahern et al., 1997; Brillantes et al., 1994; Chen et al., 1994; Ma et al., 1995) and participates in coupled gating of clusters of RyRs in lipid bilayers (Marx et al., 1998; Marx et al., 2001). In this study, we have addressed the determinants of FKB12/RyR1 interaction using SPR. Previously, SPR has examined the interaction of RyR1 with recombinant DHPR constructs (O’Reilly et al., 2002) or calsequestrin (Herzog et al., 2000), but this is the first study to measure equilibrium binding kinetics.

ELISA binding assays to determine the relationship between FKB12 and the open/closed status of RyR1 showed that RyR1 bound less FKB12 at Ca^{2+} concentrations that are associated with activation (opening) of RyR1— that is, 1 μM and 10 μM Ca^{2+} (enhanced by the addition of 1 mM ATP)— compared with RyR inhibition with EGTA (<0.01 μM Ca^{2+}) and 1 mM Ca^{2+}. Ruthenium red increased the binding of RyR1 to FKB12, relative to that of the unmodulated control, whereas caffeine had no effect, suggesting that the RyR1 preparation, stripped of endogenous FKB12, was in the open state. [3H]Ryanodine binding assays confirmed the open and closed state of the RyR1 and were appropriately modulated in agreement with previous single channel analysis (Meissner et al., 1988).

SPR was used to determine equilibrium binding kinetics. To establish specificity of binding, GST was bound to the chip and used to confirm that no binding was detected to this moiety alone (Fig. 2B). FK506 effectively abolished binding over a range of RyR1 concentrations (Fig. 2C). Endogenous FKB12 was removed from RyR1 during preparation with rapamycin and was shown by western blot to be within subsaturation levels. Loss of FKB12 was confirmed by pre-mixing recombinant FKB12 with RyR1, before flow over coupled GST-FKB12, and binding was inhibited (Fig. 2D). Inhibition of 50% was obtained at a molar ratio of ~5:1. This is close to the stoichiometric ratio of 4:1 (one binding site per subunit of RyR1) and was within the appropriate range of concentrations for the interaction of the two molecules (Timerman et al., 1995). This result also confirmed that GST-FKB12 bound to the same site as free FKB12.

The Biacore method is based on binding signal (RU) being proportional to the mass of protein bound. Thus, for 100 RU of GST-FKB12 (38 kDa), the equivalent binding signal for RyR (2200 kDa) is maximally 5800 RU, although ~500 RU was observed. Binding was, however, linearly related to the RyR1 protein concentration added (Fig. 2A,C and Fig. 4A,B). Being small molecules, GST-FKB12 and the anti-GST Ab can bind within the dextran matrix of the CM3 chip, which is inaccessible to the large RyR1 molecule, resulting in a limited proportion of the total FKB12 accessible on the surface. Moreover, binding one 2200 kDa RyR1 oligomer to GST-FKB12 probably obscures neighbouring unliganded GST-FKB12 molecules.

The K_A for the closed state of the RyR was 1×10^{13/14} M^{-1}, which is ~4-5 orders of magnitude higher than for the open channel (−1×10^9 M^{-1}). K_A is derived from k_a/k_d and the increased affinity of closed RyR1 for FKB12 was due to a decreased k_d. Nevertheless, the open channel affinity was comparable to an Ab-antigen interaction so, although the open channel is more vulnerable to dissociation of FKB12, this loss would be slow. The K_A equates to K_P values of ~0.1-0.01 μM for the close state compared with ~1 μM for the open channel. A previous study derived a value of ~300 nM for K_P (Timerman et al., 1995); the slow rate of dissociation may have contributed to this lower affinity because a labelled FKB12 exchange experiment was used. In a previous study, modulators of RyR1 open and closed states had no effect on the quantity of FKB12 bound to RyR1 in GST-FKB12 pulldown assays (Bultynck et al., 2001), thus our observations reflect the discriminatory power of the SPR technique. The impact of complete loss of FKB12/12.6 from RyR1/RyR2 are changes in the stability of the open/closed state of RyR in bilayers (Ahern et al., 1997; Brillantes et al., 1994; Chen et al., 1994; Ma et al., 1995) and altered EC coupling and Ca^{2+} sparks in skeletal (Avila et al., 2003) and cardiac (Gomez et al., 2004; Prestle et al., 2001; Xiao et al., 1997) myocytes. Our data suggests that, because FKB12 dissociates slowly and retains a comparatively high affinity for the open channel, loss of FKB12 from RyR may not be a part of the normal EC coupling cycle. With regard to the pathology of heart failure, where both RyR1 (Reiken et al., 2003; Ward et al., 2003) and RyR2 (Wehrens et al., 2004) are reported to be stripped of FKBP12/12.6 by ‘hyperphosphorylation’, the latter would need to have effects over and above increasing the open state of the channel (Meissner, 2004).

The RyR1 tetramer undergoes a considerable symmetrical conformational change between the open and closed states (Serysheva et al., 1999). It is not known whether the functional open and closed states equate to the same physical conformational changes for all agents— for example, a pharmacological agent could block the channel pore without physically altering the protein structure. Here, we show that K_A segregated the channel modulators according to their effect on the open or closed states. This suggests that both the physiological and pharmacological agents studied here broadly affect the RyR in equivalent ways, altering the conformation of the protein and manifesting as a change in the dissociation rate constant, k_d, for FKB12.

In this study, we have shown that FKB12 equilibrium binding kinetics are modified by the open or closed state of RyR1. The affinity is greatest for the closed RyR1 channel and, although four to five orders of magnitude lower for the open conformation, the interaction remains at high affinity for both
channel states. The underlying cause of the change in affinity is likely to be a conformational change in the RyR affecting the FKBPs12 binding site (Zissimopoulos and Lai, 2005). In the future, it will be interesting to observe whether modulations such as phosphorylation, or mutations such as those causing malignant hyperthermia, change the RyR/FKBPs12 interaction, differently from the modulation of the open/closed conformation. In conclusion, these studies show that SPR provides a highly sensitive technique to discern changes in the interaction of modulatory proteins with RyR affecting its activation state.

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