THE GLN$^{483}$ ALA MUTATION WITHIN A PUTATIVE, PORE-LINING TRANS-MEMBRANE HELIX OF THE CARDIAC RYANODINE RECEPTOR CHANNEL ALTERS BOTH THE KINETICS OF RYANOID INTERACTION AND THE SUBSEQUENT FRACTIONAL CONDUCTANCE.

Kishani M. Ranatunga, Tracy M. Moreno-King, Bhavna Tanna, Ruiwu Wang, S.R. Wayne Chen, Luc Ruest, William Welch, Alan J. Williams.

Myocardial Systems Biology Group, National Heart and Lung Institute, Imperial College London, London SW3 6LY, United Kingdom (K.M.R., T.M.M-K., B.T., A.J.W.)

Cardiovascular Research Group, Departments of Physiology and Biophysics and of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada (R.W., S.R.W.C.)

Department of Chemistry, University of Sherbrooke, Sherbrooke, Quebec J1K 2R1, Canada (L.R.)

145 Howard Medical Sciences, Department of Biochemistry/330, University of Nevada, Reno, 89557, USA (W.W.)
Running title: Interaction of various ryanoids with Gln\textsuperscript{486}Ala of RyR2

Address correspondence to: Prof. Alan J. Williams
Myocardial Systems Biology Group, National Heart and Lung Institute, Guy Scadding Building, Imperial College London, London SW3 6LY, United Kingdom;
Tel: +44 (0)20 7351 8137
Fax: +44 (0)20 7823 3392
E-mail: a.j.williams@imperial.ac.uk

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Non-standard abbreviations: RyR, ryanodine receptor channel; CoMFA, comparative molecular field analysis; Po, open probability; IP\textsubscript{3}R, InsP\textsubscript{3} receptor channel; \textit{wt}, wild type; GPR, guanidinopropionylryanodine, 21-p-nitro, 21-p-nitrobenzoylamino-9\textalpha-hydroxyryanodine; 8\beta-amino, 8\beta-amino-9\textalpha-hydroxyryanodine; 21-amino, 21-amino-9\textalpha-hydroxyryanodine.
ABSTRACT

The specific, high affinity interaction of the plant toxin ryanodine with its molecular target the ryanodine receptor channel (RyR) has been instrumental in RyR research. Ala-scanning of putative pore regions of mouse RyR2 has highlighted the amino acid Gln 4863, predicted to lie within trans-membrane helix TM10, as an important determinant of ryanodine binding. We have investigated the effects of several ryanodine derivatives, guanidinopropionylryanodine, 21-p-nitrobenzoylamino-9α-hydroxyryanodine, 8β-amino-9α-hydroxyryanodine, and 21-amino-9α-hydroxyryanodine, with the mouse Q4863A RyR2 mutant at the single-channel level. Our results demonstrate that the rate of dissociation of all ryanoids investigated is increased by the mutation. The modification of channel function following ryanoid-binding is qualitatively similar for wt and mutant but, in several cases, single-channel conductances were increased with Q4863A. These novel findings have been interpreted within the framework of existing CoMFA studies on ryanoids. We suggest that replacement of a glutamine by an alanine residue at position 4863 causes RyR2 to simultaneously alter interactions with both ends of the ryanoid molecule.
INTRODUCTION

Ryanodine, a plant alkaloid, is a natural insecticide and pharmacological agent. It acts to alter muscle contraction via major disruption of Ca^{2+}-flux from the sarcoplasmic reticulum (Sutko et al., 1997). Ryanodine receptor channels (RyR) are the specific target for the toxin, with each receptor containing a single high affinity binding site (Sutko et al., 1997). At low concentrations (nM-µM), binding of ryanodine to RyR leads to altered channel function characterized by high single-channel open probability (Po) and reduced rate of ion permeation; the modified state (Rousseau et al., 1987; Ashley and Williams, 1990). Higher concentrations render the channel closed to ion flow (Tinker et al., 1996). Ryanodine has been instrumental in elucidating the role of RyRs in excitation-contraction coupling of muscle (Marban and Wier, 1985) and has enabled extraction and purification of RyRs from native tissue and subsequent cloning and expression (Williams et al., 2001). Its unusually tight and specific binding to RyR renders it a useful biochemical probe of the RyR structure-function relationship.

RyRs are large, tetrameric ion channel proteins (monomer ~550 kDa), located in the sarco(endo)plasmic reticulum (Sutko and Airey, 1996). They mediate the primary pathway for regulated elevation of intracellular, free Ca^{2+} in a variety of cell types (Berridge et al., 2003) thereby contributing to a wide array of physiological processes. Channel conduction has been localized to the C-terminal fifth of the protein by truncation (Bhat et al., 1997). Bioinformatics (Shah and Sowdhamini, 2001; Williams et al., 2001) and extensive mutational studies have further identified putative channel elements of RyRs (Zhao et al., 1999; Gao et al., 2000; Du et al., 2001; Chen et al., 2002; Wang et al., 2003; Wang et al., 2004; Du et al., 2004). Most recently, a 3D model of the RyR pore (Welch et al., 2004) has been proposed based on structural analogies with the known X-ray structure of the prokaryotic K^+ channel, KcsA (Doyle et al., 1998).
The high affinity ryanodine-binding site also resides in the carboxyl-terminus of RyRs based on proteolytic digest and photo-affinity labeling studies (Callaway et al., 1994; Witcher et al., 1994). Extensive Ala-scanning of these putative pore-forming domains of RyRs has identified several residues that abolish $[^3]$H]ryanodine binding (Zhao et al., 1999; Gao et al., 2000; Du et al., 2001; Chen et al., 2002; Wang et al., 2003; Wang et al., 2004; Du et al., 2004). Those that retain caffeine-sensitivity, implying lack of gross distortion of structure, but abolish ryanodine-sensitivity indicate a clear effect on the ryanodine binding site (Wang et al., 2003). Several residues in the putative re-entrant pore loop, Gly 4826, Gly 4828, Asp 4829, and one in the putative pore-lining, inner helix, Gln 4863, fall into this category.

The related InsP3 receptors (IP3Rs) also functionally serve as intracellular Ca$^{2+}$-release channels and show significant sequence similarity to RyRs in predicted pore regions (Shah and Sowdhamini, 2001). Unlike RyRs, IP3Rs cannot bind ryanodine (Perez et al., 1997; Ramos-Franco et al., 1998) suggesting some level of structural difference. Based on the alignment of the two families of Ca$^{2+}$-release channels, presented by Shah and Sowdhamini (Shah and Sowdhamini, 2001), residues 4826, 4828, and 4829 are conserved as Gly, Gly, and Asp respectively in both RyRs and IP3Rs. However, amino acid 4863 is conserved through all RyRs as the polar, aliphatic Gln but non-conservatively mutated to the hydrophobic, aromatic Phe throughout IP3Rs. Thus both mutational analysis and sequence comparison highlight this residue as a prime determinant in ryanodine binding.

RyR binding affinity and subsequent ion permeation are also affected by structural changes to ryanodine (Sutko et al., 1997; Tinker et al., 1996). To further understand the ryanodine/RyR2 interaction we have investigated the effects of the Q4863A RyR2 mutation on the interaction with several well-characterized ryanodine derivatives (ryanoids). Our studies reveal that the mutation not only alters the binding affinity between toxin and channel but is also able to influence ion flow when the toxin is bound.
MATERIALS AND METHODS

Materials

Phosphatidylethanolamine was supplied by Avanti Polar Lipids, Inc., USA. Ryanodine was purchased from Agrisystems International, USA and \(^{3} \text{H}\)ryanodine from Amersham Biosciences, UK. Ryanoids (21-p-nitrobenzoylamino-9\(\alpha\)-hydroxyryanodine, 21-amino-9\(\alpha\)-hydroxyryanodine, 8\(\beta\)-amino-9\(\alpha\)-hydroxyryanodine, guanidinopropionylryanodine) were synthesized as described previously (Welch et al., 1997). All ryanoids were stored as stock solutions in 50-100 % ethanol or methanol at -20°C. All other reagents were of the highest grade and obtained from VWR International, USA or Sigma-Aldrich Co., USA.

Site-directed mutagenesis, DNA transfection, and protein purification

The methodology followed that of Wang et al. (Wang et al., 2003). Briefly, the single-point, Q4863A mutant was introduced into mouse RyR2 by PCR. Either \(wt\) or Q4863A mutant DNA was transfected into HEK-293 cells via CaPO\(_{4}\) precipitation. Recombinant channels were purified from cell lysates using sucrose density gradient centrifugation and \(^{3}\text{H}\)ryanodine binding.

Planar phospholipid bilayers

Planar phospholipid bilayers were formed from a 35 mg.ml\(^{-1}\) suspension of phosphatidylethanolamine in \(n\)-decane and single RyR channels were incorporated as described previously (Tinker et al., 1996). The recording solution consisted of 600 mM KCl and 20 mM HEPES titrated to pH 7.4 with KOH (adding 10 mM K\(^{+}\)). The \(trans\) chamber is held at virtual ground and the cis chamber clamped to the desired electrical potential. RyR2 channels incorporate in a fixed orientation such that the cytosolic face of the channel is
exposed to the cis chamber and the luminal to the trans chamber. Channels were activated with up to 200 µM EMD41000 added to the cytosolic side (McGarry and Williams, 1994). Channels were ryanoid-modified by addition of the relevant concentration of the ryanoid to the cis chamber. All experiments were carried out at room temperature, 21±2 °C.

Single-channel data acquisition and analysis

Single channel currents were filtered with low-pass, 8-pole Bessel filters at 1 kHz and 5 kHz then sampled at 20 kHz, with a PCI-6036e AD board (National Instruments, USA), for acquisition and viewing using Acquire4.0/5.0.1 (Bruxton Corporation, USA). Data were exported from Review4.0 (Bruxton Corporation, USA) as ASCII text files and re-formatted with ActivePerl5.8 (ActiveState, Canada). Single-channel current amplitudes were estimated from non-linear, least-squares fitting of a sum of Gaussian distributions to all points histograms constructed from the 5 kHz filtered trace using Mathematica5.0 (Wolfram Research, USA). Modified rates of ion flux induced by the interaction of a ryanoid were quantified as a fractional conductance; defined as the conductance of the modified state expressed as a proportion of conductance in the absence of ryanoid.

Dwell times in the ryanoid modified states were measured directly off-screen from the 1 kHz filtered trace with Review4.0. Ryanoid dwell time distributions were plotted as normalized histograms and non-linear, least-squares fit with single exponential distributions, as a first approximation, using Mathematica5.0. Other statistical analyses were performed in a standard spreadsheet package (Excel2002, Microsoft Corporation, USA). Representative traces were filtered and exported with Tac4.1.5 (Bruxton Corporation, USA) and annotated in a standard graphics package (CorelDraw8, Corel Corporation, USA).
RESULTS

It has been established that the Q4863A mutation markedly reduces RyR2 affinity for ryanodine by apparently increasing its dissociation rate without effect on the single-channel conductance of the modified state (Wang et al., 2003). To confirm that transitions between periods of modified and normal channel gating reflect dissociation of the ryanoid, we have investigated the consequences of removing ryanodine and changing ryanodine concentration. Following perfusion of ryanodine from Q4863A RyR2 channels further modified events were not observed (n=4, Figure 1B). This indicates that ryanodine does not remain bound to the channel during normal gating in the presence of the toxin. Increasing the concentration of ryanodine 10-fold (from 1 to 10 µM) increases the probability of the channel being in the modified state (Figure 1D and E). Dwell time analysis of the modified and unmodified states—assuming a simple, bimolecular interaction scheme (Tanna et al., 1998)—reveals no change in the rate of dissociation (360±72 µs⁻¹ at 1 µM and 321±37 µs⁻¹ at 10 µM; holding potential +20 mV; n=5) but an approximate 10-fold increase in the rate of association (200±100 µs⁻¹ at 1 µM and 2900±1800 µs⁻¹ at 10 µM; holding potential +20 mV; n=5). The simplest hypothesis that fits these observations of reversibility on washout and concentration dependence is that the mutant channel exhibits reversible kinetics in the presence of ryanodine analogous to that observed with the wt channels and certain other ryanoids (see below). Explicitly the modified state represents the channel with ryanoid bound and the unmodified state the channel without ryanoid.

To further explore the molecular mechanism of the functional differences caused by Q4863A, we have investigated the effects on this mutant of ryanodine and various ryanoids: guanidinopropionylryanodine (GPR), 21-p-nitrobenzoylamino-9α-hydroxyryanodine (21-p-nitro), 8β-amino-9α-hydroxyryanodine (8β-amino) and 21-amino-9α-hydroxyryanodine (21-amino).
Earlier investigations using sheep wt RyR2 and a range of ryanoids (Tinker et al., 1996; Tanna et al., 1998; Tanna et al., 2002; Tanna et al., 2003) have established some basic features of the interactions of ryanoids with RyR2, the subsequent function of the ryanoid-RyR2 complex and the influence of ryanoid structure on these processes. Both ryanoid association and dissociation are influenced by structural features of the ligand with greater effects on the latter. In addition, the probability of interaction is modified by trans-membrane holding potential; as potential is taken from negative to positive values, the rate of association of the ligand increases while its rate of dissociation decreases. Formation of the ryanoid-RyR2 complex results in modified rates of ion flux through the channel - expressed as a fraction of the unmodified conductance. This fractional conductance of the modified state is again determined by structural features of the toxin.

The Q4863A mutation markedly increases the reversibility of ryanoid interaction with RyR2

Ryanodine, GPR, and 21-p-nitro elicit long-lived, modified states in sheep wt RyR2 that have been described as ‘irreversible’ within the time-frame of single-channel experiments (>>10^3 s) (Tinker et al., 1996). Interactions of these ryanoids with mouse wt RyR2 result in modified conductance states with values of fractional conductance consistent with those observed in the sheep channel; ryanodine and GPR each give rise to a single modified state (Figure 2A and C) while three different fractional conductance states are observed with 21-p-nitro (labeled i, ii and iii in Figure 3A-C). Similarly, rates of dissociation of these ryanoids from mouse wt RyR2 are extremely slow with no dissociation events observed at a holding potential of +40 mV. When a holding potential at which dissociation is likely to be favored (-60 mV) was imposed we observed no dissociation of either ryanodine or GPR, however at this and more positive potentials we did, in a proportion of channels,
observe dissociation of 21-p-nitro from fractional conductance states i (three of four) and ii (four of six) (unpublished observations).

The interaction of ryanodine, 21-p-nitro and GPR with the Q4863A mutant channel appears altered compared to wt. At a holding potential of +40 mV several modified events are observed during recordings with ryanodine (Figure 2A) and 21-p-nitro (Figure 3D). At a holding potential of +40 mV the interaction kinetics of ryanodine (Wang et al., 2003) and 21-p-nitro (Table 1) with Q4863A RyR2 are shifted to timescales amenable to analysis. Dwell times in the GPR-modified state of Q4863A are considerably longer than those of ryanodine and 21-p-nitro precluding detailed analysis. However this ryanoid does dissociate slowly at +40 mV and within seconds at -60 mV (Ranatunga, K.M., Moreno-King, T.M., Tanna, B., Wang, R., Chen, S.R.W., Ruest, L., Welch, W., Williams, A.J. Manuscript in preparation).

Figure 2B and D show representative modifications of the mouse wt channel by 8β-amino and 21-amino respectively at a holding potential of +40 mV. Unlike ryanodine, 21-p-nitro and GPR, both of these ryanoids display reversible kinetics under these conditions, although dwell times with 8β-amino are noticeably longer than those of 21-amino. In keeping with our observations with irreversible ryanoids, dwell times for these ryanoids with the Q4863A mutant are distinctly shorter (~10⁻¹ s) than those seen for the wt RyR2 (Figure 2B and D). Quantification of the kinetics of these interactions of ryanoids with Q4863A requires the development of alternative methods of analysis and is not investigated further here.

**Effects of trans-membrane holding potential on the dwell times and distribution of states induced by 21-p-nitro modification of the Q4863A mutant**

21-p-nitro, on interaction with RyR2 (wt or Q4863A), induces one of three, modified states of differing fractional conductance; i, ii, iii in order of decreasing conductance (Figure
3). The enhanced rates of ryanoid dissociation observed in the Q4863A mutant have enabled us to quantify the probability of occurrence of these three states, the dwell times in the different states and the influence of holding potential on these parameters. Table 1 demonstrates that a clear difference exists in the likelihood of occurrence of the three fractional conductance states with $P(\text{ii}) > P(\text{i}) > P(\text{iii})$. Probabilities of occurrence of the three states do not vary with trans-membrane holding potential (Table 1).

Estimates of dwell times of the three fractional conductance states with Q4863A reveal that each state has distinct kinetics (Table 2). Durations increase from ii to i to iii and this order remains the same with changing voltage. Durations within each state generally increase with increasing trans-membrane potential (note the lower number of events for state iii at 60 mV).

*Fractional conductances are altered on ryanoid interaction with the Q4863A mutant as compared to wt RyR2*

Ryanoid-modified states exhibit lower single-channel conductance as compared to the unmodified channel, quantified as the fractional conductance parameter. Ryanodine and GPR invariably result in the occurrence of a single fractional conductance state on interaction with the wt channel (Figure 2A and C). Interaction of 21-p-nitro with the wt channel results in the occurrence of one of three conductance states (Figure 3). Modification of the Q4863A mutant by these ryanoids is similar in that each interaction results in a single fractional conductance state (Figures 2 and 3). However, fractional conductances are significantly increased for GPR and state ii of 21-p-nitro (Table 3).

On interaction with wt RyR2 8β-amino and 21-amino channels display noisy ryanoid-bound states that reveal, on closer inspection, a rapid flickering between two, energetically similar conformations (Figure 2B and D). This behavior is retained when 8β-amino is bound
to the Q4863A mutant but the fractional conductances of both the resultant states are increased (Table 3). In contrast, the 21-amino-Q4863A RyR2 complex exhibits only a single state not significantly different in fractional conductance to that of the lower conductance conformation observed with wt RyR2 (Table 3).
DISCUSSION

The Q4863A mutation increases the rate of dissociation from RyR2 of several ryanoids

This study clearly demonstrates that mutation of Gln to Ala at position 4863 of RyR2 alters the kinetics of interactions with the tested ryanoids by increasing the rate of dissociation from the receptor. Rates of ryanoid dissociation from RyR2 are influenced by structural features of the ligand, with ryanoids broadly classified as irreversible or reversible based on their likelihood of dissociation within the time-frame of a single-channel experiment. These studies demonstrate that, irrespective of the likelihood of dissociation from the wt channel, Q4863A increases the probability of dissociation. In addition, the Q4863A mutant reveals that 3, nominally irreversible states induced by 21-p-nitro, have markedly different rates of dissociation.

Qualitatively, we can construct orders of ‘reversibility’, from longest to shortest dwell times in ryanoid-bound states for mouse RyR2:

wt: ryanodine~GPR~21-p-nitro(i)~21-p-nitro(iii)>21-p-nitro(ii)>>8β-amino ~21-amino,
Q4863A: GPR >21-p-nitro(iii)>ryanodine~21-p-nitro(i)>21-p-nitro(ii)>>8β-amino~21-amino.

The similarity of these two sequences suggests that the shift in ryanoid-RyR2 affinity due to Q4863A is unidirectional and probably similar in extent for all ryanoids tested. This supports the notion that the same mechanism underlies the increase in ryanoid-reversibility induced by Q4863A.

We interpret the modified conductance states reported in this communication to represent the RyR2 channel with a single ryanoid molecule bound at a high affinity site. This interpretation is based upon the observation that dissociation constants determined in macroscopic binding experiments with intact wt sarcoplasmic reticulum vesicles for 10-O-succinoylryanodol, ryanodol and 21-amino-9α-hydroxyryanodine are comparable with the
apparent dissociation constants derived from analysis of single wt channel kinetics of modified states induced by these ryanoids (Tanna et al., 2003). In addition, as is the case for high affinity binding of $[^3H]$-ryanodine to populations of RyR channels, the probability of ryanoid interaction resulting in a modified conductance state of an individual RyR2 channel, is dependent upon channel open probability (Tanna et al., 1998). However it should be noted that a long-lasting modified-conductance state has been observed in the presence of a high concentration of ryanodine (10 $\mu$M) in an individual RyR1 channel, containing a mutation in a putative calcium binding motif, that shows no detectable binding of $[^3H]$-ryanodine (Fessenden et al., 2004).

*The type of ryanoid-modified state is hardly altered by Q4863A.*

Ryanoid-binding to RyRs leads to either single ‘quiet’ or noisy modified states. The latter reflects a rapid flickering between energetically similar RyR-ryanoid conformations with different ion-handling properties (Tanna et al., 2002). In addition, some ryanoids, such as 21-p-nitro (Figure 3) and 10-O-succinoylryanodol (Tanna et al., 2003), elicit single states of differing fractional conductance on each interaction. The type of modified state, induced by the various ryanoids examined here, remains broadly similar between wt and Q4863A. This is in agreement with the hypothesis that the differing states are due to different conformations of the ryanoid (Tanna et al., 2001) and suggests little influence by the protein (or at least Gln4863) in this behavior. A notable exception is the interaction of 21-amino which is altered from a noisy state with wt to a single state (Figure 2D), similar to the wt $\alpha$ state, with Q4863A. The interaction of 8$\beta$-amino also shows a greater preference towards the $\alpha$ state though both states are seen (Figure 2B). One possibility is that the higher fractional conductance state (less likely to occur with Q4863A) is better stabilized by Gln than Ala at position 4863.
Ion translocation in the modified state is changed for some ryanoids by Q4863A

Although the type of ryanoid modified state and increase in rates of dissociation appear similar for various ryanoids between wt and Q4863A, the effects on subsequent ion-handling display stark differences. Whilst many modified states display no change in fractional conductance with the mutant, several states (GPR single state; 21-p-nitro ii; 8β-amino α and β) exhibit increased ion flux. There is no obvious relationship between these changes and wt fractional conductance or locus of structural difference in the ryanoid. Indeed, only one of three states induced by 21-p-nitro is altered. However, it is interesting to note that, in general, where fractional conductances are changed, they are increased with the Q4863A mutation; the β state of 8β-amino is the only observed exception.

It is an open question whether the lowered RyR ion flux brought about by ryanoid-interaction is due to: a) induction of unique RyR/ryanoid conformations, further complicated by the possibility that ryanoids may bind within the pore and directly influence ion permeation, or b) stabilization of short-lived or infrequent, RyR configurations that exist in the absence of ryanoid. On interaction with certain ryanoid conformations, Q4863A either renders the RyR/ryanoid conformation more permissive to ion flow than wt (a) or favors alternate, higher fractional conductance RyR states (b).

Structural features of ryanoids that determine interaction with RyRs

Quantitative analysis of correlations between structural features of ryanoids and their functional effects (Sutko et al., 1997) has identified the pyrrole locus, and neighboring isopropyl groups as primary determinants of binding whilst changes at position 10 and 21, at the opposite end of the molecule, influence fractional conductance. The asymmetric
distribution of polar groups on the ryanodine molecule lead to clearly defined hydrophobic and polar faces (Sutko et al., 1997).

Our observations that the Q4863A mutation can alter both binding kinetics and subsequent ion conduction suggest that the mutation might affect and sense changes in the two opposite ends of the ryanoid molecule.

Structural implications of a Gln to Ala mutation in a trans-membrane helix

The polar residues Gln and Asn, though rare in trans-membrane proteins, are highly conserved and show a propensity to be buried in the middle of the bilayers at interfaces (Lear et al., 2003). Through hydrogen-bonding they can mediate helix-helix association. Indeed, Gln4863 resides towards the middle of an otherwise hydrophobic, predicted trans-membrane helix.

Ala-scanning suggests that this residue may be pore-lining (Wang et al., 2003) and a recent modeling study of the RyR2 pore places it at the top of a putative aqueous cavity (Welch et al., 2004). Molecular dynamics studies with this model highlight a robust intersubunit hydrogen bond between Gln 4863 of the inner helix and the backbone of a neighboring putative filter region (Rheault, S. and Welch, W. Personal communication). Although there are limitations (Welch et al., 2004), this demonstrates that the involvement of Gln4863 in intersubunit interactions is plausible and suggests the filter region as a potential target.

It is interesting to note that a similarly positioned Asn in some inwardly rectifying potassium channels (Asp in others), has prime importance in determining the binding affinity of intracellular blocking cations (Lu and MacKinnon, 1995).
Speculation on the ryanodine binding site of RyR2

Several lines of circumstantial evidence are consistent with the proposal that ryanodine binds within the ion conduction pathway of RyR2 within a wide vestibule: a) ryanoids bind to the open state of the channel (Tanna et al., 1998), b) the voltage-dependence of ryanoid interaction is evident with all tested ryanoids but stronger with cationic ones suggesting interaction of the ryanoid with the voltage drop across the membrane (Tanna et al., 2000), c) ryanoid-binding affects the location of binding sites of pore-blockers (Tanna et al., 2001). We note that there is no definitive evidence, to date, that can distinguish between a pore-bound ryanoid interaction and an allosteric effect.

It has been suggested that the hydrophobic face of ryanoids sits against the hydrophobic wall of the putative aqueous cavity with the pyrrole locus submerged in a cleft towards the selectivity filter and the 9, 10 end hanging towards the cytoplasmic end of RyR (Wang et al., 2003). It is tempting to speculate that direct interactions with Gln 4863 hold the molecule in place. The data presented in this study suggests that Gln 4863 might sense opposite ends of the molecule. Either this could be by indirect effects such as rigid-body or hinge motions of this helix, or Gln 4863 residues of other subunits may make direct interactions. There are several targets for interactions on the polar face of the toxin. This might suggest a ‘cross’-pore positioning of the ryanodine molecule within the cavity.

Alternatively, considering an allosteric model, loss of the polar group at position 4863 may have effects on inter- and intra-subunit interactions. This would cause indirect changes to the ryanodine binding site and its role in altering conduction when toxin is bound.

The major novel finding of this study is that the Q4863A mutation of mouse RyR2 is able to influence the rate of ion flow through some ryanoid/RyR2 complexes in addition to a clear, and apparently uniform, increase in dissociation rate. Interpreted within the framework of an extensive ComFA analysis, this indicates that residues at the 4863 position, putatively
located in the centre of a TM helix, can sense spatially distinct regions of the ryanoid molecule.
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FOOTNOTES

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Address reprint requests to: Prof. Alan J. Williams, Myocardial Systems Biology Group, National Heart and Lung Institute, Imperial College London, London SW3 6LY, United Kingdom.
FIGURE LEGENDS

Figure 1. Validation of reversibility of the Q4863A RyR2 ryanodine interaction. Representative traces, filtered to 1 kHz, of a single Q4863A channel at a holding potential of +40 mV. A. normal gating, B. following addition of 1 μM ryanodine, C. following washout of ryanodine. Concentration dependence of ryanodine Q4863A RyR2 interaction at a holding potential of +20 mV D. in the presence of 1 μM ryanodine E. in the presence of 10 μM ryanodine.

Figure 2. The kinetics of ryanoid interaction are altered by the Q4863A mutation of RyR2. Representative traces, filtered to 1 kHz, of single RyR2 channels at a holding potential of +40 mV in the presence of A. ryanodine, B. 8β-amino (only the association of 8β-amino with wt is shown in this figure), dissociation occurred a few s afterwards, C. GPR, and D. 21-amino (modified events with Q4863A are shown as a montage of 6 separate events rather than a continuous recording). C- closed. O- open. Modified states are labeled M for ryanodine and GPR, α and β for 8β-amino and 21-amino (Table 1).

Figure 3. The interaction of Q4863A and 21-p-nitro displays all three states observed irreversibly with wt in single recordings. Representative single channel traces, filtered to 1 kHz, of RyR2 channels at a holding potential of +40 mV in the presence of 21-p-nitro. A. wt with state i of 21-p-nitro, B. wt with state ii of 21-p-nitro, C. wt with state iii of 21-p-nitro, D. Q4863A with all three states of 21-p-nitro.
Table 1. The probability of occurrence of 21-p-nitro fractional conductance states, P(state), in the Q4863A mutant does not vary with altered trans-membrane holding potential. Data were pooled from four or more experiments in each case.

<table>
<thead>
<tr>
<th>21-p-nitrobenzoyl-amino-9α-hydroxyryanodine</th>
<th>P(state) %</th>
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<tbody>
<tr>
<td></td>
<td>Q4863A</td>
</tr>
<tr>
<td></td>
<td>20 mV</td>
</tr>
<tr>
<td>i</td>
<td>20</td>
</tr>
<tr>
<td>ii</td>
<td>72</td>
</tr>
<tr>
<td>iii</td>
<td>8</td>
</tr>
<tr>
<td>Total no. of events</td>
<td>683</td>
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</table>
Table 2. The dwell times of the three 21-p-nitro states with Q4863A differ and increase with trans-membrane holding potential.

Data were pooled from four or more experiments in each case. The standard error quoted is the uncertainty estimate from the fitting routine (See Methods).

<table>
<thead>
<tr>
<th>21-p-nitrobenzoyl-amino-9α-hydroxyryanodine</th>
<th>τ±SE (N) (ms)</th>
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<tr>
<td></td>
<td>20 mV</td>
</tr>
<tr>
<td>i</td>
<td>1880±70 (136)</td>
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<tr>
<td>ii</td>
<td>181±6 (490)</td>
</tr>
<tr>
<td>iii</td>
<td>7700±400 (57)</td>
</tr>
</tbody>
</table>
Table 3. The fractional conductances of some modified states are shifted with Q4863A.

All measurements were made at a holding potential of +40 mV. Significant differences from wt, as calculated by pairwise Students t-test, are indicated by asterices: * P<0.05, ** P<0.01.

On one occasion, a ~50 % state was observed with GPR/wt RyR2 not included in analysis here. SDOM is the standard deviation of the mean from N experiments.

<table>
<thead>
<tr>
<th>Ryanoid</th>
<th>Name of state</th>
<th>Fractional conductance %</th>
<th>Mean±SDOM (N)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>wt</td>
<td>Q4863A</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>M</td>
<td>52±2 (4)</td>
<td>60±2 (3)*</td>
<td></td>
</tr>
<tr>
<td>Guanidino-propionyl ryanodine</td>
<td>M</td>
<td>5.7±0.4 (5)</td>
<td>18±2 (4)**</td>
<td></td>
</tr>
<tr>
<td>8β-amino, 9α-hydroxyryanodine</td>
<td>β</td>
<td>56±1 (11)</td>
<td>51±1 (4)**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>35.3±0.7 (11)</td>
<td>40.2±0.9 (10)**</td>
<td></td>
</tr>
<tr>
<td>21-amino, 9α-hydroxyryanodine</td>
<td>β</td>
<td>47.1±0.7 (5)</td>
<td>not observed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>37.4±0.7 (5)</td>
<td>39±1 (3)</td>
<td></td>
</tr>
<tr>
<td>21-p-nitro-amino-9α-hydroxyryanodine</td>
<td>i</td>
<td>54±4 (4)</td>
<td>57±2 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ii</td>
<td>22.8±0.6 (5)</td>
<td>43±2 (5)**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>iii</td>
<td>12.3±0.5 (2)</td>
<td>17.5±0.9 (4)*</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3