

The Gln⁴⁸⁶³Ala 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

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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. Alanine scanning of putative pore regions of mouse RyR2 has highlighted the amino acid Gln4863, 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-9 α -hydroxyryanodine, 8 β -amino-9 α -hydroxyryanodine, and 21-amino-9 α -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 after ryanoid binding is qualitatively similar for wild-type and mutant, but in several cases, single-channel conductances were increased with Q4863A. These novel findings have been interpreted within the framework of existing comparative molecular field analysis 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.

Ryanodine, a plant alkaloid, is a natural insecticide and pharmacological agent. It acts to alter muscle contraction via major disruption of Ca²⁺ flux from the sarcoplasmic reticulum (Sutko et al., 1997). Ryanodine receptor channels (RyRs) are the specific target for the toxin, with each receptor containing a single high-affinity binding site (Sutko et al., 1997). At low concentrations (from nanomolar to micromolar), binding of ryanodine to RyR leads to altered channel function characterized by high single-channel open probability and reduced rate of ion permeation: the modified state (Rousseau et al., 1987; Ashley and Williams, 1990). Higher concentra-

tions 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 Ca²⁺ 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 mu-

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ABBREVIATIONS: RyR, ryanodine receptor channel; wt, wild type; GPR, guanidinopropionylryanodine, 21-*p*-nitro, 21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine; 8 β -amino, 8 β -amino-9 α -hydroxyryanodine; 21-amino, 21-amino-9 α -hydroxyryanodine.

tational studies have further identified putative channel elements of RyRs (Zhao et al., 1999; Gao et al., 2000; Du et al., 2001, 2004; Chen et al., 2002; Wang et al., 2003, 2004). Most recently, a 3D model of the RyR pore (Welch et al., 2004) has been proposed derived from 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 derived from proteolytic digest and photo-affinity labeling studies (Callaway et al., 1994; Witcher et al., 1994). Extensive alanine scanning of these putative pore-forming domains of RyRs has identified several residues that abolish [³H]ryanodine binding (Zhao et al., 1999; Gao et al., 2000; Du et al., 2001, 2004; Chen et al., 2002; Wang et al., 2003, 2004). Those that retain caffeine sensitivity, implying a 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, Gly4826, Gly4828, Asp4829, and one in the putative pore-lining, inner helix, Gln4863, fall into this category.

The related InsP3 receptors (IP3Rs) also serve functionally as intracellular Ca²⁺-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. On the basis of the alignment of the two families of Ca²⁺-release channels, presented by Shah and Sowdhamini (2001), residues 4826, 4828, and 4829 are conserved as glycine, glycine, and aspartic acid, respectively, in both RyRs and IP3Rs. However, amino acid 4863 is conserved through all RyRs as the polar, aliphatic glutamine but nonconservatively mutated to the hydrophobic, aromatic phenylalanine 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 (Tinker et al., 1996; Sutko et al., 1997). 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 (Alabaster, AL). Ryanodine was purchased from Agri-systems International (Wind Gap, PA), and [³H]ryanodine was from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Ryanoids (21-*p*-nitrobenzoylamino-9 α -hydroxyryanodine, 21-amino-9 α -hydroxyryanodine, 8 β -amino-9 α -hydroxyryanodine, and guanidinopropionylryanodine) were synthesized as described previously (Welch et al., 1997). All ryanoids were stored as stock solutions in 50 to 100% ethanol or methanol at -20°C. All other reagents were of the highest grade and were obtained from VWR (West Chester, PA) or Sigma-Aldrich (St. Louis, MO).

Site-Directed Mutagenesis, DNA Transfection, and Protein Purification. The methodology followed that of Wang et al. (2003). In brief, the single-point Q4863A mutant was introduced into mouse

RyR2 by polymerase chain reaction. Either wt or Q4863A mutant DNA was transfected into human embryonic kidney 293 cells via Ca₃(PO₄)₂ precipitation. Recombinant channels were purified from cell lysates using sucrose density gradient centrifugation and [³H]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 was held at virtual ground, and the *cis* chamber was 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 μ M 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 \pm 2°C.

Single-Channel Data Acquisition and Analysis. Single-channel currents were filtered with low-pass, eight-pole Bessel filters at 1 and 5 kHz and then sampled at 20 kHz with a PCI-6036e AD board (National Instruments, Austin, TX) for acquisition and viewing using Acquire 4.0/5.0.1 (Bruerton Corporation, Seattle, WA). Data were exported from Review 4.0 (Bruerton) as ASCII text files and were reformatted with ActivePerl 5.8 (ActiveState, Vancouver, BC, Canada). Single-channel current amplitudes were estimated from nonlinear, least-squares fitting of a sum of Gaussian distributions to all-points histograms constructed from the 5-kHz filtered trace using Mathematica 5.0 (Wolfram Research, Champaign, IL). 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 Review 4.0. Ryanoid dwell-time distributions were plotted as normalized histograms and nonlinear least-squares fit with single exponential distributions, as a first approximation, using Mathematica 5.0. Other statistical analyses were performed in a standard spreadsheet package (Excel 2002; Microsoft Redmond, WA). Representative traces were filtered and exported with Tac 4.1.5 (Bruerton) and annotated in a standard graphics package (CorelDraw 8, Corel Corporation, Ottawa, ON, Canada).

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 investigated the consequences of removing ryanodine and changing ryanodine concentration. After perfusion of ryanodine from Q4863A RyR2 channels further modified events were not observed (*n* = 4; Fig. 1, A–C). 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 μ M) increases the probability of the channel being in the modified state (Fig. 1, D 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 \pm 72 μ s⁻¹ at 1 μ M and 321 \pm 37 μ s⁻¹ at 10 μ M; holding potential, +20 mV; *n* = 5) but an

approximate 10-fold increase in the rate of association ($200 \pm 100 \mu\text{s}^{-1}$ at $1 \mu\text{M}$ and $2900 \pm 1800 \mu\text{s}^{-1}$ at $10 \mu\text{M}$; holding potential, $+20 \text{ mV}$; $n = 5$). 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 represents the channel without ryanoid.

To further explore the molecular mechanism of the functional differences caused by Q4863A, we investigated the effects on this mutant of ryanodine and various ryanoids: guanidinopropionylryanodine (GPR), 21-*p*-nitrobenzoyl-amino-9 α -hydroxyryanodine (21-*p*-nitro), 8 β -amino-9 α -hydroxyryanodine (8 β -amino), and 21-amino-9 α -hydroxyryanodine (21-amino).

Earlier investigations using sheep wt RyR2 and a range of ryanoids (Tinker et al., 1996; Tanna et al., 1998, 2002, 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 dissociation. 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 as 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. Structural features of the toxin again determine the fractional conductance of the modified state.

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 ($\gg 10^3 \text{ s}$) (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 (Fig. 2, A and C) whereas three different fractional conductance states are observed with 21-*p*-nitro (labeled i, ii, and iii in Fig. 3, A–C). Likewise, rates of dissociation of these ryanoids from mouse wt RyR2 are extremely slow, with no dissociation events observed at a holding potential of $+40 \text{ mV}$. 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 the dissociation of 21-*p*-nitro from fractional conductance states i (three of four) and ii (four of six).

The interaction of ryanodine, 21-*p*-nitro, and GPR with the Q4863A mutant channel seems altered compared with wt. At a holding potential of $+40 \text{ mV}$, several modified events are observed during recordings with ryanodine (Fig. 2A) and 21-*p*-nitro (Fig. 3D). At a holding potential of $+40 \text{ mV}$, the interaction kinetics of ryanodine (Wang et al., 2003) and 21-*p*-nitro (Table 1) with Q4863A RyR2 are shifted to time scales 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 $+40 \text{ mV}$ and within seconds at -60 mV .

Figure 2, B and D, shows representative modifications of the mouse wt channel by 8 β -amino and 21-amino, respectively, at a holding potential of $+40 \text{ mV}$. Unlike ryanodine, 21-*p*-nitro, and GPR, these ryanoids display reversible kinetics under these conditions, although dwell times with 8 β -amino are noticeably longer than those of 21-amino. In keep-

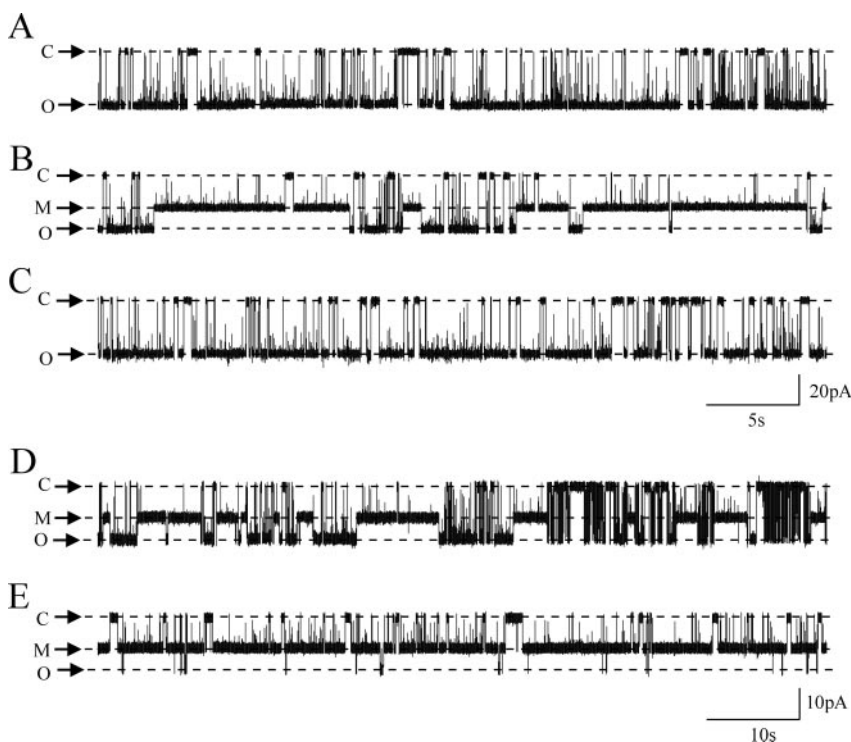


Fig. 1. Validation of reversibility of the Q4863A RyR2 ryanodine interaction. Representative traces, filtered to 1 kHz , of a single Q4863A channel at a holding potential of $+40 \text{ mV}$, normal gating (A), after the addition of $1 \mu\text{M}$ ryanodine (B), and after washout of ryanodine (C). Concentration-dependence of ryanodine Q4863A RyR2 interaction at a holding potential of $+20 \text{ mV}$ in the presence of $1 \mu\text{M}$ ryanodine (D) and in the presence of $10 \mu\text{M}$ ryanodine (E). C, closed; O, open; M, modified.

ing with our observations with irreversible ryanoids, dwell times for these ryanoids with the Q4863A mutant are distinctly shorter ($\sim 10^{-1}$ s) than those seen for the wt RyR2 (Fig. 2, B 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 (K. M. Ranatunya, T. M. Moreno-King, B. Tanna, R. Wang, S. R. W. Chen, L. Ruest, W. Welch, and A. J. Williams, manuscript in preparation).

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, upon interaction with RyR2 (wt or Q4863A), induces one of three modified states of differing fractional conductance: i, ii, and iii, in order of decreasing conductance (Fig. 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 $P(ii) > P(i) > P(iii)$. 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 Compared with wt RyR2. Ryanoid-modified states exhibit lower single-channel conductance compared with the unmodified channel, quantified as the fractional conductance parameter. Ryanodine and GPR invariably result in the occurrence of a single fractional conductance state upon interaction with the wt channel (Fig. 2, A and C). Interaction of 21-*p*-nitro with the wt channel results in the occurrence of one of three conductance states (Fig. 3). Modification of the Q4863A mutant by

these ryanoids is similar in that each interaction results in a single fractional conductance state (Figs. 2 and 3). However, fractional conductances are significantly increased for GPR and state ii of 21-*p*-nitro (Table 3).

Upon interaction with wt RyR2, 8 β -amino and 21-amino channels display noisy ryanoid-bound states that reveal, on closer inspection, a rapid flickering between two energetically similar conformations (Fig. 2, B and D). This behavior is retained when 8 β -amino is bound to the Q4863A mutant, but the fractional conductances of both of 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 from 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 glutamine to alanine at position 4863 of RyR2 alters the kinetics of interactions with the tested ryanoids by increasing the rate of dissociation from the receptor. Structural features of the ligand influence rates of ryanoid dissociation from RyR2; ryanoids are broadly classified as irreversible or reversible on the basis of 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 three 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)>>8 β -amino~21-amino; and Q4863A, GPR > 21-*p*-nitro(iii)>ryanodine~21-*p*-nitro(i)>21-*p*-nitro(ii)>>8 β -amino~21-amino. The similarity of these two sequences suggests that the shift in ryanoid-RyR2 affinity caused by Q4863A is unidirectional and probably is similar in extent for all ryanoids tested. This supports the notion that

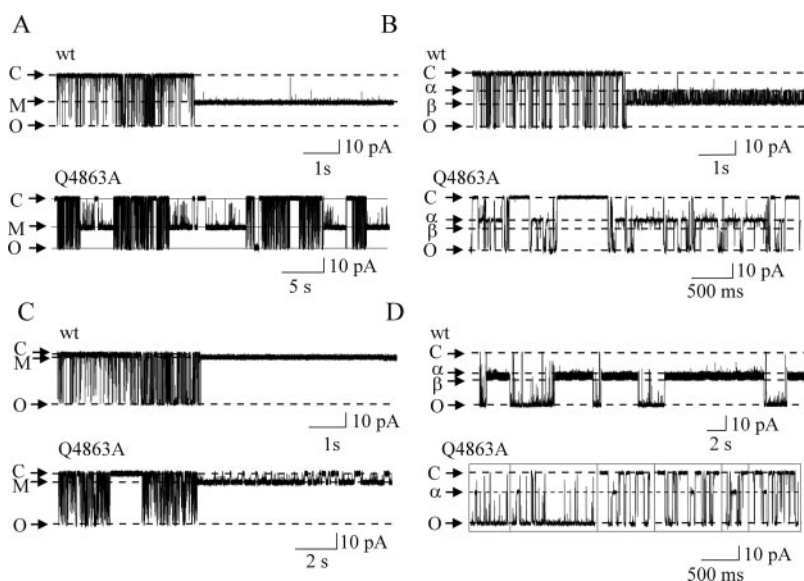


Fig. 2. The kinetics of ryanoid interaction are altered by the Q4863A mutation of RyR2. Representative traces, filtered to 1 kHz, of single RyR2 channels at a holding potential of +40 mV in the presence of ryanodine (A), 8 β -amino (only the association of 8 β -amino with wt is shown in this figure),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

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 derived from the observation that dissociation constants determined in macroscopic binding experiments with intact wt sarcoplasmic reticulum vesicles for 10-*O*-succinylryanodol, ryanodol, and 21-amino-9 α -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 on 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 μ 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 (Fig. 3) and 10-*O*-succinylryanodol (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 caused by 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 (Fig. 2D), similar to the wt α state, with Q4863A. The interaction of 8 β -amino also shows a greater preference toward the α state, although both states are seen (Fig. 2B). One possibility is that the higher fractional conductance state (less likely to occur with Q4863A) is better stabilized by glutamine than by alanine 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 seem similar for various ryanoids between wt and Q4863A, the effects on subsequent ion-handling display stark differences. Whereas many modified states display no change in fractional conductance with the mutant, several states (GPR single state; 21-*p*-nitro ii; 8 β -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 8 β -amino is the only observed exception.

It is an open question whether the lowered RyR ion flux

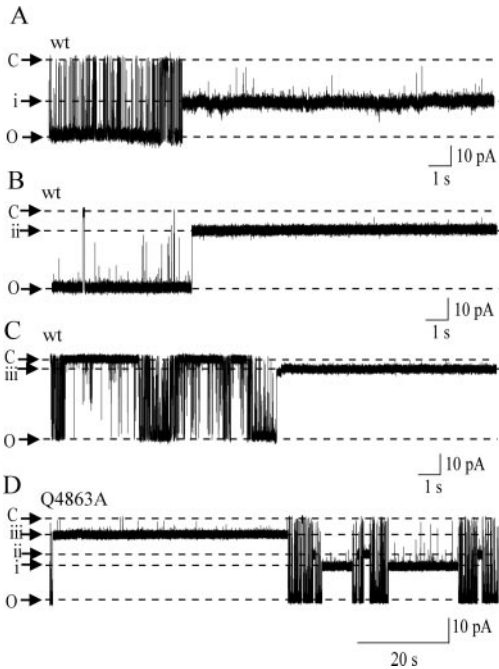


Fig. 3. The interaction of Q4863A and 21-*p*-nitro displays all three states observed irreversibly with wt in single recordings. Representative single-channel traces, filtered to 1 kHz, of RyR2 channels at a holding potential of +40 mV in the presence of 21-*p*-nitro. A, wt with state i of 21-*p*-nitro. 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.

21- <i>p</i> -Nitrobenzoyl-amino-9 α -hydroxyryanodine	Q4863A P(state)		
	20 mV	40 mV	60 mV
	%		
i	20	14	16
ii	72	71	75
iii	8	11	9
Total no. of events	683	322	247

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 *Materials and Methods*). Numbers shown in parentheses are *n* values.

21- <i>p</i> -Nitrobenzoyl-amino-9 α -hydroxyryanodine	$\tau \pm$ S.E.		
	20 mV	40 mV	60 mV
	ms		
i	1880 \pm 70 (136)	4900 \pm 300 (55)	6900 \pm 300 (40)
ii	181 \pm 6 (490)	340 \pm 20 (230)	543 \pm 9 (185)
iii	7700 \pm 400 (57)	62,000 \pm 7000 (37)	29,000 \pm 4000 (22)

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