

Two Rings of Negative Charges in the Cytosolic Vestibule of Type-1 Ryanodine Receptor Modulate Ion Fluxes

Le Xu,* Ying Wang,* Dirk Gillespie,[†] and Gerhard Meissner*

*Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599;

and [†]Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, Illinois 60612

ABSTRACT The tetrameric ryanodine receptor calcium release channels (RyRs) are cation-selective channels that have pore architecture similar to that of K⁺ channels. We recently identified, in close proximity to the selectivity filter motif GGGIG, a conserved luminal DE motif that has a critical role in RyR ion permeation and selectivity. Here, we substituted three aspartate residues (D⁴⁹³⁸, D⁴⁹⁴⁵, D⁴⁹⁵³) with asparagine and four glutamate residues (E⁴⁹⁴², E⁴⁹⁴⁸, E⁴⁹⁵², E⁴⁹⁵⁵) with glutamine hypothesized to line the cytosolic vestibule of the skeletal muscle RyR (RyR1). Mutant single channel properties were determined using the planar lipid bilayer method. Two mutants (D⁴⁹³⁸N, D⁴⁹⁴⁵N) showed a reduced K⁺ ion conductance, with D⁴⁹³⁸N also exhibiting a reduced selectivity for Ca²⁺ compared to K⁺. The cytosolic location of D⁴⁹³⁸ and D⁴⁹⁴⁵ was confirmed using the polycation neomycin. Both D⁴⁹³⁸N and D⁴⁹⁴⁵N exhibited an attenuated block by neomycin to a greater extent from the cytosolic than luminal side. By comparison, charge neutralization of luminal loop residues (D⁴⁸⁹⁹Q, E⁴⁹⁰⁰N) eliminated the block from the luminal but not the cytosolic side. The results suggest that, in addition to negatively charged residues on the luminal side, rings of four negative charges formed by D⁴⁹³⁸ and D⁴⁹⁴⁵ in the cytosolic vestibule determine RyR ion fluxes.

INTRODUCTION

Ryanodine receptor ion channels (RyRs) regulate various cellular functions by releasing Ca²⁺ from intracellular Ca²⁺ stores (1–3). The RyRs are cation-selective channels that have a high ion conductance for both monovalent (~800 pS with 250 mM K⁺ as conducting ion) and divalent (~150 pS with 50 mM Ca²⁺) cations, yet do so selectively ($P_{Ca}/P_K \sim 7$) (4–6). Since RyR is highly cation selective, it is likely that negative charges line the pore of this channel. The location of these charges in RyR has been probed using large organic and inorganic positively charged molecules. Monovalent cations produced well-resolved voltage-dependent blocking events from the cytosolic side (7,8), whereas polycations such as neomycin or ruthenium red were capable of blocking RyR channels from both sides (9–12). These studies indicated the presence of negatively charged residues on both the cytosolic and luminal ends of the conductance pathway of the RyRs.

Mammalian RyRs have ~70% sequence identity, with the highest level of identity in the C-terminal, pore-forming region. Hydropathy analysis initially suggested between 4 and 12 transmembrane segments per RyR subunit (13,14). More recent studies have indicated the presence of six to eight transmembrane helices in each RyR1 subunit (15). The most C-terminal membrane-spanning segment of RyR has been proposed to be the inner helix of the pore (16), and amino acid residues linking the two C-terminal membrane-spanning segments are believed to correspond to the pore-

forming segment of K⁺ channels. Sequence comparison and mutagenesis studies have also suggested that the RyR ion channels have a pore structure similar to that of K⁺ channels (whose structure is known) (17–22). In RyR, the residues between the two C-terminal membrane-spanning segments are lumenally located (23) and have a predicted pore helix followed by an amino acid motif (GGGIG) that is similar to the selectivity filter motif (TV/IGYG) of K⁺ channels (24).

However, there exist significant functional and sequence differences. First, K⁺ channels selectively conduct K⁺ ions, whereas RyRs have a high ion conductance for both monovalent and divalent cations. Second, the RyR pore has a minimum radius of ~3.5 Å as estimated from the relative permeability of organic cations (25), compared to a radius of ~2 Å for K⁺ channels (26). Third, mutagenesis and single channel measurements have shown that a conserved RyR luminal DE motif in close proximity to the selectivity filter motif GGGIG has a critical role in RyR ion permeation and selectivity (21); the two amino acids are not fully conserved or even absent in K⁺ channels.

In this study, we mutated seven negatively charged amino acid residues hypothesized to line the cytosolic vestibule of RyR1. Charge neutralization of two acidic residues (D⁴⁹³⁸ and D⁴⁹⁴⁵) closely following the most C-terminal membrane-spanning segment reduced RyR1 K⁺ ion permeation, with D⁴⁹³⁸N also exhibiting a reduced Ca²⁺ selectivity. The cytosolic location of these two residues was confirmed using the polycationic RyR blocker neomycin. Substitution of D⁴⁹³⁸ or D⁴⁹⁴⁵ with asparagine attenuated block by neomycin to a greater extent from the cytosolic than luminal side. The results suggest that, in addition to negatively charged residues on the luminal side, rings of negative

Submitted August 11, 2005, and accepted for publication October 4, 2005.

Le Xu and Ying Wang contributed equally to this work.

Address reprint requests to Gerhard Meissner, Tel.: 1-919-966-5021; Fax: 1-919-966-2852; E-mail: meissner@med.unc.edu.

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0006-3495/06/01/443/11 \$2.00

doi: 10.1529/biophysj.105.072538

charges in the cytosolic vestibule have a role in maintaining the high RyR1 ion fluxes.

EXPERIMENTAL PROCEDURES

Materials

Human embryonic kidney (HEK) 293 cells were obtained from ATCC, [³H]ryanodine from Perkin Elmer Life Sciences (Boston, MA), unlabeled ryanodine from Calbiochem (La Jolla, CA), Fluo-4-AM from Molecular Probes (Eugene, OR), complete protease inhibitors from Roche (Indianapolis, IN), and phospholipids from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of analytical grade.

Construction of mutant cDNAs

For construction of RyR1 mutants, a fragment (*Clal/XbaI*, 14443/15276) of the full length rabbit RyR1 cDNA subcloned into pBluescript vector (20) was used as template for mutagenesis. Single base changes were introduced by *Pfu*-turbo polymerase-based chain reaction using mutagenic oligonucleotides and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The complete mutated sequences were confirmed by DNA sequencing and subcloned back into the *Clal* and *XbaI* sites of pCMV5 vector containing *EcoRI/XbaI* fragment of RyR1. Mutated full-length expression plasmids were prepared by ligation of three fragments (*Clal/XhoI*, *XhoI/EcoRI*, *EcoRI/XbaI* containing the mutated sequence) and expression vector pCMV5 (*Clal/XbaI*) as described (20). Nucleotide and amino acid numbering are as described (13).

Expression of full-length wild-type and mutant RyRs in HEK 293 cells

RyR1 cDNAs were transiently expressed in HEK 293 cells using FuGENE 6 (Roche Applied Science) as described (21). Crude membrane fractions and proteoliposomes containing the purified 30 S wild-type and mutant RyR1 ion channels were prepared in presence of protease inhibitors (21).

Ca²⁺ release measurements

Cellular Ca²⁺ release in response to caffeine was determined with the fluorescence Ca²⁺ indicator dye Fluo-4 (21). HEK 293 cells transfected with cDNA encoding wild-type or mutant RyR1s were grown for 48 h on glass coverslips and, after washing with phosphate-buffered saline, were loaded with 5 μM Fluo-4-AM for 1 h at 37°C in Krebs-Ringer-Henseleit (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 6 mM glucose, 1.2 mM MgCl₂, 2 mM CaCl₂, and 25 mM HEPES, pH 7.4). After rinsing with KRH buffer to remove nonhydrolyzed fluorophore, cells were transferred to a recording chamber with KRH buffer and Fluo-4 fluorescence was recorded and analyzed using a Photon Technology International Deltascan system attached to an inverted microscope (TE 300; Nikon) and ImageMaster program (Photon Technology International, Lawrenceville, NJ). Caffeine responsiveness of cells was recorded by rapid addition of freshly made caffeine solution to a final concentration of 10 mM.

[³H]Ryanodine binding

Ryanodine is a widely used probe of channel activity because of its preferential binding to the open channel state (2,27). B_{max} values of [³H]ryanodine binding were determined by incubating membranes for 4 h at 24°C with a saturating concentration of [³H]ryanodine (40 nM) in 20 mM imidazole, pH 7.0, 0.6 M KCl, 0.15 M sucrose, 5 mM oxidized glutathione, 20 μM leupeptin, 200 μM Pefabloc, and 100 μM Ca²⁺. To determine Ca²⁺ dependence of [³H]ryanodine binding, membranes were incubated for 20 h

at 24°C with 2.5 nM [³H]ryanodine in 20 mM imidazole (pH 7.0), 0.15 M sucrose, 250 mM KCl, protease inhibitors, 5 mM oxidized glutathione, and free Ca²⁺ concentrations ranging 0.5–500 μM. Samples were diluted with eight volumes of ice-cold water and placed on Whatman (Brentford, UK) GF/B filters preincubated with 2% polyethyleneimine in water. Filters were washed with three 5 ml aliquots of ice-cold 100 mM KCl, 1 mM KPipes (pH 7.0) solution. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [³H]ryanodine. Nonspecific binding was determined using a 1000–2000-fold excess of unlabeled ryanodine.

Single channel recordings

Single channel measurements were performed using Mueller-Rudin type planar lipid bilayers containing a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (25 mg of total phospholipid/ml *n*-decane) (28). Proteoliposomes containing the purified RyRs were added to the *cis* (sarcoplasmic reticulum (SR) cytosolic side) chamber of a bilayer apparatus and fused in the presence of an osmotic gradient (250 mM *cis* KCl/20 mM *trans* KCl in 20 mM KHepes, pH 7.4, with 2–20 μM Ca²⁺ and 1 mM ATP in the *cis* chamber). After appearance of channel activity, *trans* (SR luminal side) KCl concentration was increased to 250 mM to prevent further fusion of proteoliposomes. The *trans* side of the bilayer was defined as ground. The large cytosolic regulatory region of the channels faced the *cis* chamber in a majority (>98%) of the recordings (28). Electrical signals were filtered at 2 kHz (0.5 kHz for Ca²⁺ currents at 0 mV), digitized at 10 kHz, and analyzed as described (12).

To determine permeability ratios, single channel activities were recorded in symmetrical 250 mM KCl solution with 10 mM Ca²⁺ on the *trans* side and the reversal potential (*E*_{rev}) was measured. The permeability ratio of Ca²⁺/K⁺ ions (*P*_{Ca}/*P*_K) was calculated using a modified form of the Goldman–Hodgkin–Katz equation:

$$E_{rev} = -\frac{RT}{F} \ln \left\{ [K]^{\frac{1}{2}} \times \left([K] + 4 \frac{P_{Ca}}{P_K} [Ca] \right)^{-\frac{1}{2}} \right\}, \quad (1)$$

where [Ca] is the concentration of Ca²⁺ ions on the *trans* side, and [K] is the concentration of K⁺ ions on the *cis* and *trans* side.

Kinetic analysis of neomycin-induced partial block in ryanodine-modified channels

Cytosolic neomycin caused a substate at positive but not negative holding potentials, and luminal neomycin caused a substate at negative but not positive holding potentials in ryanodine-modified RyR2 (11). The same results were obtained for ryanodine-modified wild-type and mutant RyR1s (see Figs. 3–5, *C* and *D*). The subconductance was analyzed by a simple biomolecular scheme (11).

For ryanodine-modified channels the closed events were rare. The occurrence of substates could therefore be assessed by the open probability (*P*_o), which was determined by 50% threshold analysis with cursors set on the ryanodine-modified state (see Figs. 3–5, *O*) and substate (see Figs. 3–5, *s*–):

$$P_s = 1 - P_o = 1 / \{ 1 + K_{50} / [\text{neomycin}] \}, \quad (2)$$

where *K*₅₀ is the concentration of neomycin at which *P*_s was 0.5. This approach also yields dwell times of modified state (*T*_o) and substate (*T*_s)

$$k_{on} = 1/T_o \quad (3)$$

$$k_{off} = 1/T_s \quad (4)$$

$$K_D = (k_{off}/k_{on})[\text{neomycin}], \quad (5)$$

where *k*_{on} and *k*_{off} are association and dissociation rate constants of neomycin and *K*_D is the dissociation constant. The relationships between *K*_D and holding potential were fitted using the following equation:

$$K_D(V) = K_D(0)\exp[-z_{\text{total}}(FV/RT)], \quad (6)$$

where z_{total} is the total effective gating charge of block induced by neomycin, F is the Farady constant, and V is holding potential.

Biochemical assays and data analysis

Free Ca^{2+} concentrations were obtained by including in the solutions the appropriate amounts of Ca^{2+} and EGTA as determined using the stability constants and the computer program published by Schoenmakers et al. (29). Free Ca^{2+} concentrations were verified with the use of a Ca^{2+} -selective electrode.

Results are given as mean \pm SE. Significance of differences in the data ($p < 0.05$) was determined using Student's t -test.

RESULTS

The amino acid sequences of the predicted pore-forming regions of the skeletal muscle (type 1) ryanodine receptor are shown in Fig. 1. Shown are the luminal loop linking the two most C-terminal transmembrane-spanning helices (A) and the most C-terminal transmembrane-spanning segment (C-terminal TM) and an extension by 22 amino acid residues (B). The luminal loop has an amino acid motif (GGGIG) similar to the sequence TVGYG that determines the selectivity of the bacterial K^+ channel KcsA (24). In a recent study, we mutated the conserved glutamate and aspartate residues of the luminal loop to asparagine and glutamine residues (indicated in *bold* in Fig. 1 A). We found that neutralizing the charge of two luminal RyR1 residues (D^{4899} , E^{4900}) immediately after the GGGIG motif reduced K^+ ion conductance and selectivity for Ca^{2+} compared to K^+ (21). Here we consider whether neutralization of negative charges following the sequence of predicted C-terminal TM (indicated in *bold* in Fig. 1 B) would have similar effects.

We substituted three aspartate residues (D^{4938} , D^{4945} , D^{4953}) with asparagine and four glutamate residues (E^{4942} , E^{4948} ,

E^{4952} , E^{4955}) with glutamine. The mutants were expressed in HEK 293 cells, and their expression levels and functional properties were assessed by immunoblot analysis, cellular Ca^{2+} release, and [^3H]ryanodine binding, as previously described (21). Single channel recordings were performed to determine the ion permeation properties of the mutants.

Functional properties of RyR1 pore mutants

Immunoblot analysis showed that the seven RyR1 mutants were expressed in HEK 293 cells at a level comparable to wild-type (Fig. 1S, Supplemental Data). The ability of the mutants to release Ca^{2+} in response to the Ca^{2+} -releasing drug caffeine was determined in a cellular fluorescence assay using Fluo-4. Fig. 2 shows three representative Ca^{2+} release curves for vector-, wild-type-RyR1-, and RyR1- D^{4938} N-transfected cells. Vector-transfected cells showed no caffeine response, indicating that HEK 293 cells express endogenous RyRs at background levels. Similar time courses and peak amplitudes of Ca^{2+} release were observed for wild-type and RyR1- D^{4938} N (Fig. 2), as well as for RyR1- D^{4945} N, - E^{4948} Q, - E^{4952} Q, and - E^{4955} Q (Fig. 2S, Supplemental Data, Table 1). Two mutants (RyR1- E^{4942} Q and - D^{4953} N) displayed a background signal comparable to vector-transfected cells (Table 1).

The expression of functional RyR1 mutant proteins was also probed by determining their ability to bind [^3H]ryanodine (2,27). Four of the seven mutants (RyR1- D^{4938} N, - D^{4945} N, - E^{4952} Q, - E^{4955} Q) bound [^3H]ryanodine (Table 1) and showed Ca^{2+} dependence of [^3H]ryanodine binding close to that of wild-type RyR1 (Fig. 3S, Supplemental Data). The two mutants (E^{4942} Q and D^{4953} N) that failed to show a caffeine response also exhibited a loss of high-affinity [^3H]ryanodine binding. RyR1- E^{4948} Q exhibited a caffeine response but showed greatly reduced levels of [^3H]ryanodine

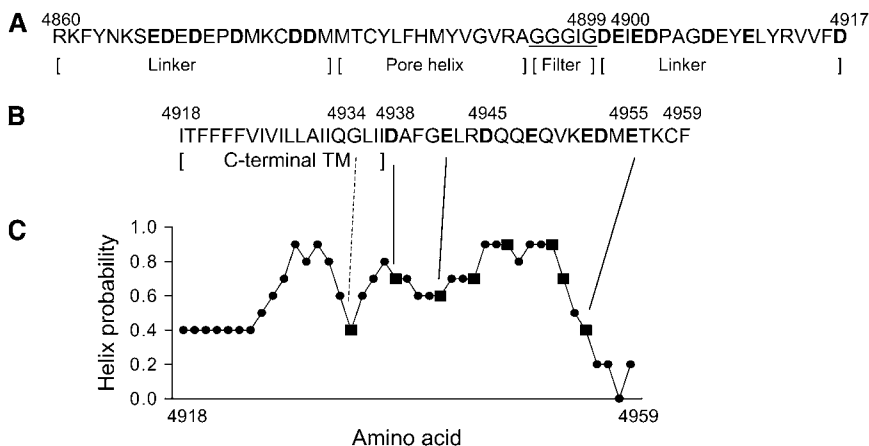


FIGURE 1 Pore-lining sequences of RyR1. Shown are the amino acid sequences of luminal loop spanning the two most C-terminal transmembrane-spanning segments (A) and the most C-terminal transmembrane-spanning segment (C-terminal TM) and an extension by 22 residues (B). C-terminal TM corresponds to TM4 of the four membrane-spanning model of Takeshima et al. (13) and TM10 (without N-terminal amino acid residues VVFD) of the 12 membrane-spanning model of Zorzato et al. (14) used by Du et al. (15) to describe an 8 membrane-spanning model. (C) Helical probability of putative C-terminal TM and 22 residues immediately after C-terminal TM was calculated, using the PROF protein sequence analysis program (42,43). In A, the putative RyR1 pore helix, selectivity filter, and linker regions are shown. Bold indicates negatively charged amino acid residues mutagenized to asparagine or glutamine (21, this study). Underlined amino acids indicate residues analogous to the selectivity filter of K^+ channels (26).

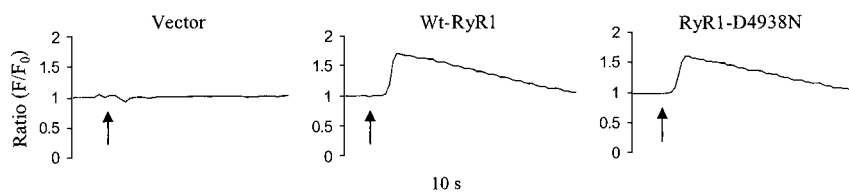


FIGURE 2 Caffeine-induced Ca^{2+} release in HEK 293 cells expressing wild-type RyR1 and RyR1-D⁴⁹³⁸N. HEK 293 cells were transfected with pCMV5 vector, wild-type RyR1 and RyR1-D⁴⁹³⁸N cDNAs. The fluorescence intensity of Fluo-4 loaded cells was measured before and after addition of 10 mM caffeine. Arrows indicate the addition of caffeine. Representative traces are shown.

binding (Table 1), which indicated loss of function during membrane isolation.

K⁺ conductance of RyR1 mutant channels

The ion permeation properties of the mutant RyR1 ion channels that showed retention of function were determined by fusing proteoliposomes containing the purified 30 S channel complexes with planar lipid bilayers. Single channels were recorded in 250 mM KCl medium on both sides of the lipid bilayer with K⁺ as current carrier. Single channel currents can be efficiently recorded in KCl solutions because RyR does not noticeably conduct anions like Cl[−] and conducts monovalent cations more efficiently than Ca²⁺. The *cis* (cytosolic) bilayer chamber contained micromolar activating Ca²⁺ (2–20 μM) and 1 mM ATP to increase single channel activity. To ascertain retention of pharmacological regulation, cytosolic Ca²⁺ concentrations were reduced to 0.1 μM and increased to 100 μM . We found that the mutants that retained both caffeine response and [³H]ryanodine binding exhibited a Ca²⁺ dependence similar to that of wild-type RyR1 (Fig. 4S, Supplemental Data).

In symmetrical 250 mM KCl, wild-type RyR1 had a mean conductance of ~ 800 pS (Fig. 3, Table 2). Removal of the negative charge in RyR1-D⁴⁹³⁸N (Fig. 4) significantly decreased K⁺ conductance to 520 ± 6 pS (Table 2). The K⁺ conductance of D⁴⁹⁴⁵N decreased to a lesser extent to 737 ± 11 pS (Table 2). RyR1-E⁴⁹⁵²Q (not shown) and RyR1-E⁴⁹⁵⁵Q (Table 2) showed a K⁺ conductance not

significantly different from wild-type. We conclude that the negatively charged side chain of D⁴⁹³⁸ (and to a lesser extent D⁴⁹⁴⁵) influences K⁺ permeation of RyR1.

Cation selectivity of RyR1 mutants

RyRs permeate Ca²⁺ with a higher selectivity than K⁺ (4–6). To determine the ion selectivity of the RyR1 mutants, current-voltage curves were recorded in 250 mM symmetric KCl with and without 10 mM Ca²⁺ in the *trans* (SR lumenal) bilayer chamber. The single channel currents and current-voltage relationships of wild-type (*solid symbols*) and RyR1-D⁴⁹³⁸N (*open symbols*) are compared (see Fig. 6 A) in the presence (*squares*) and absence (*circles*) of 10 mM *trans* Ca²⁺. In the absence of Ca²⁺, the K⁺ currents showed linear voltage dependence. Addition of 10 mM Ca²⁺ to the *trans* chamber reduced currents at elevated positive and negative potentials. At 0 mV, a Ca²⁺ current of -2.8 pA for wild-type RyR1 and reduced current of -0.8 pA for D⁴⁹³⁸N were measured (Table 2). With the addition of Ca²⁺, the reversal potentials (E_{rev}) for wild-type and D⁴⁹³⁸N were shifted to $+9.5$ and $+5.3$ mV, respectively, which gave permeability ratios of Ca²⁺/K⁺ ($P_{\text{Ca}}/P_{\text{K}}$) of 7.0 and 3.3. By comparison, RyR1-D⁴⁹⁴⁵N and E⁴⁹⁵⁵Q (Table 2) and E⁴⁹⁵²Q (not shown) had E_{rev} and $P_{\text{Ca}}/P_{\text{K}}$ values not substantially different from wild-type.

Location of RyR1 mutants

The lumenal or cytosolic location of the RyR1 mutants, in relation to the selectivity filter, was assessed using neomycin. This large polycation has been shown to induce a voltage-dependent block in the open state of the cardiac ryanodine receptor (RyR2) from both ends of the channel (9–11). We examined the blocking effects of neomycin on wild-type and the two mutants that exhibited a reduced K⁺ conductance (RyR1-D⁴⁹³⁸N, -D⁴⁹⁴⁵N) as well as one mutant (RyR1-E⁴⁹⁵⁵Q) that had ion permeation not significantly different from wild-type. We also tested RyR1-D⁴⁸⁹⁹Q and -E⁴⁹⁰⁰N, which are located on the lumenal loop of RyR1 (Fig. 1 A) and exhibit a reduced K⁺ conductance and Ca²⁺ selectivity (Table 2).

To facilitate detection of neomycin-blocking events, wild-type RyR1 and mutant ion channels were modified using the neutral plant alkaloid ryanodine. Ryanodine forms in single channel recordings a subconductance (30) that is largely

TABLE 1 Ca²⁺-release and [³H]ryanodine-binding properties of wild-type and mutant RyR1s

Name	Caffeine-induced Ca ²⁺ release	[³ H]Ryanodine binding
Vector	—	—
Wild-type RyR1	+++	+++
D ⁴⁹³⁸ N	+++	+++
E ⁴⁹⁴² Q	—	—
D ⁴⁹⁴⁵ N	+++	+++
E ⁴⁹⁴⁸ Q	+++	+
E ⁴⁹⁵² Q	+++	+++
D ⁴⁹⁵³ N	—	—
E ⁴⁹⁵⁵ Q	+++	+++

(+++) indicates caffeine-induced Ca²⁺ release and [³H]ryanodine binding activities comparable to wild-type RyR1; (+) indicates reduced activity; and (—) indicates no activity.

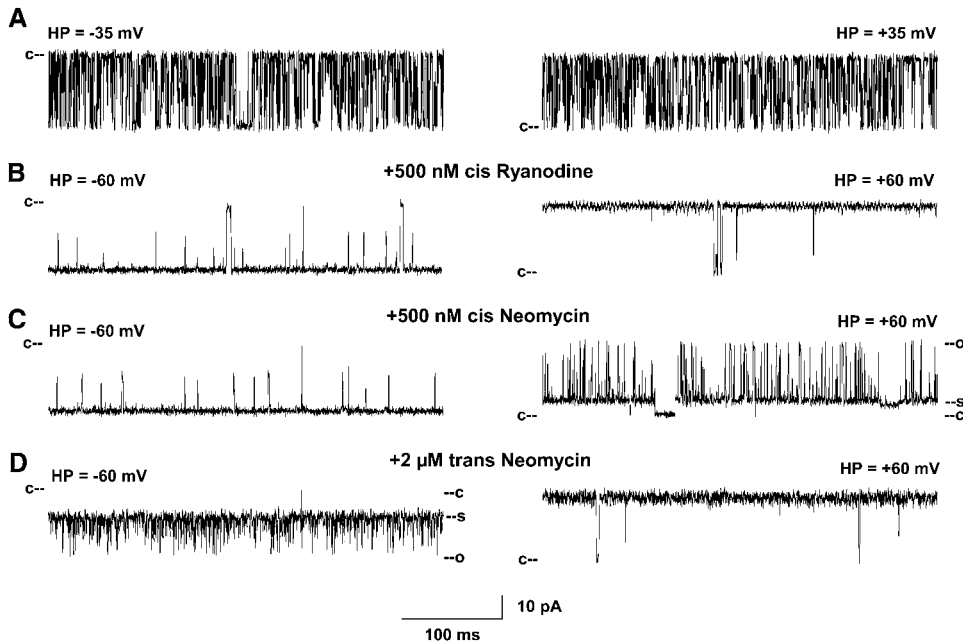


FIGURE 3 Single channel recordings of wild-type RyR1. (A) Single channel currents were recorded at indicated holding potentials in symmetrical 250 mM KCl with 2–20 μM Ca^{2+} and 1 mM ATP in the *cis* chamber as downward (*left*) or upward (*right*) inflections from the closed state (*c*–). (B–D) After addition of 500 nM ryanodine (B) and in subsequent presence of 500 nM *cis* (C) or 2 μM *trans* neomycin (D). In C and D, *c* and *o* represent the open and closed ryanodine-modified states; *s* represents the neomycin-induced substate.

insensitive to regulation by Ca^{2+} and ATP and other effectors. Moreover, neomycin does not permeate the ryanodine-modified RyR (10). Figs. 3–5 show that addition of ryanodine resulted in the formation of similarly prolonged channel openings in wild-type, D⁴⁹³⁸N, and D⁴⁸⁹⁹Q single channels. Ryanodine induced subconductances in wild-type and mutant channels, corresponding to $\sim 60\%$ of the respective full conductances, with the exception of RyR1-D⁴⁹³⁸N, which formed a subconductance corresponding to 75% of its full conductance (Table 2).

Neomycin induced a partial, voltage-dependent block in both ryanodine-modified wild-type RyR1 and mutants. When added to the *cis* (cytosolic) chamber of the bilayer apparatus, block was observed in wild-type at positive holding potentials (Fig. 3 and Fig. 6 B). The probability of the partial block (P_s) increased with neomycin concentration (Fig. 7 A). No blocking events were observed at negative holding potentials. Neomycin was considerably less effective in blocking RyR1-D⁴⁹³⁸N from the *cis* side at positive holding potentials (Figs. 4 and 7 A). When added to the *trans*

TABLE 2 Single channel properties of wild-type and mutant RyR1s

Parameter	wild-type	D ⁴⁸⁹⁹ Q	E ⁴⁹⁰⁰ N	D ⁴⁹³⁸ N	D ⁴⁹⁴⁵ N	E ⁴⁹⁵⁵ Q
γ_{K+} (pS)	$801 \pm 7(17)^{\dagger}$	$164 \pm 4(10)^{\dagger*}$	$505 \pm 3(3)^{\dagger*}$	$520 \pm 6(9)^*$	$737 \pm 11(6)^*$	$812 \pm 7(4)$
$I_{\text{Ca}^{2+}}$ at 0 mV (pA)	$-2.8 \pm 0.1(7)^{\dagger}$	$-0.4 \pm 0.1(4)^{\dagger*}$	$-2.0 \pm 0.2(7)^{\dagger*}$	$-0.8 \pm 0.1(4)^*$	$-2.0 \pm 0.2(5)^*$	$-2.7 \pm 0.2(3)$
E_{rev} (mV)	$9.5 \pm 0.2(6)^{\dagger}$	$1.9 \pm 0.1(4)^{\dagger*}$	$6.8 \pm 0.4(5)^{\dagger*}$	$5.3 \pm 0.3(4)^*$	$9.0 \pm 0.6(5)$	$10.7 \pm 0.7(3)$
$P_{\text{Ca}}/P_{\text{K}}$	7.0^{\dagger}	1.0^{\dagger}	4.5^{\dagger}	3.3	6.5	8.3
$\gamma_{K+ (+\text{Ry})}$ (pS)	$467 \pm 19(8)$	$105 \pm 2(9)^*$	$296 \pm 4(9)^*$	$378 \pm 8(6)^*$	$429 \pm 7(9)$	$457 \pm 5(4)$
$\gamma_{K+ (+\text{Ry})}/\gamma_{K+ (-\text{Ry})}$	0.58	0.62	0.59	0.75*	0.60	0.56
+ <i>cis</i> Neomycin at 70 mV						
$\gamma_{\text{sub} (+\text{Ry}, +\text{Neo})}/\gamma_{K+ (+\text{Ry})}$	$0.16 \pm 0.01(10)$	$0.37 \pm 0.04(6)^*$	$0.23 \pm 0.01(8)^*$	$0.49 \pm 0.04(3)^*$	$0.47 \pm 0.01(3)^*$	$0.15 \pm 0.02(3)$
$k'_{\text{on}} (\mu\text{M}^{-1} \text{ms}^{-1})$	$5.2 \pm 0.8(7)$	$3.2 \pm 0.7(3)$	$2.7 \pm 0.3(3)$	$0.1 \pm 0.1(3)^*$	$2.2 \pm 0.2(4)^*$	$3.5 \pm 0.3(3)$
$k_{\text{off}} (\text{ms}^{-1})$	$0.5 \pm 0.1(6)$	$1.4 \pm 0.1(3)^*$	$0.5 \pm 0.1(3)$	$0.8 \pm 0.1(3)^*$	$2.1 \pm 0.1(4)^*$	$0.3 \pm 0.1(3)$
$K_{50} (\mu\text{M})$	0.1	0.5	0.2	11.1	0.9	0.1
Z_{total}	$1.1 \pm 0.1(10)$	$1.0 \pm 0.1(3)$	$1.0 \pm 0.1(3)$	$0.4 \pm 0.1(3)^*$	$1.2 \pm 0.1(4)$	$1.1 \pm 0.1(7)$
+ <i>trans</i> Neomycin at –60 mV						
$\gamma_{\text{sub} (+\text{Ry}, +\text{Neo})}/\gamma_{K+ (+\text{Ry})}$	$0.39 \pm 0.01(8)$	†	†	$0.50 \pm 0.01(4)^*$	$0.38 \pm 0.01(4)$	$0.41 \pm 0.01(6)$
$k'_{\text{on}} (\mu\text{M}^{-1} \text{ms}^{-1})$	$1.6 \pm 0.2(5)$	†	†	$0.7 \pm 0.2(4)^*$	$0.6 \pm 0.2(4)^*$	$0.7 \pm 0.1(5)^*$
$k_{\text{off}} (\text{ms}^{-1})$	$1.4 \pm 0.4(5)$	†	†	$2.3 \pm 0.2(4)^*$	$2.3 \pm 0.2(4)^*$	$2.1 \pm 0.1(5)^*$
$K_{50} (\mu\text{M})$	0.8	†	†	3.5	3.1	2.4
Z_{total}	$-0.6 \pm 0.1(4)$	†	†	$-0.7 \pm 0.1(4)$	$-0.6 \pm 0.1(4)$	$-0.6 \pm 0.1(7)$

* $p < 0.05$ compared to wild-type.

†From Wang et al. (21).

‡Substate was not detected.

k_{off} was determined at 0.1 μM *cis* and 0.5 μM *trans* neomycin.

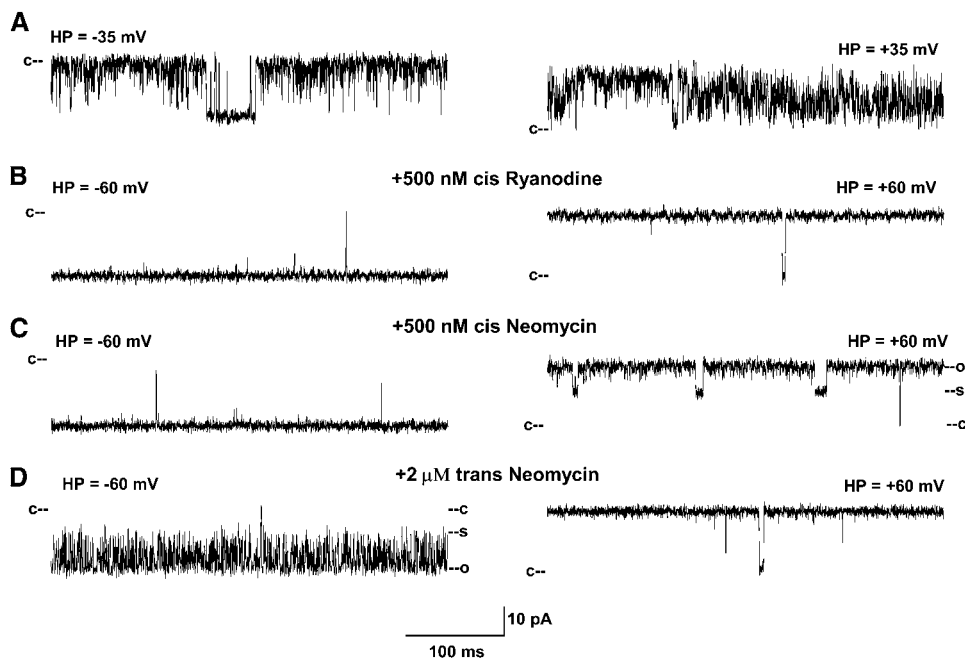


FIGURE 4 Single channel recordings of RyR1-D⁴⁹³⁸N. (A) Single channel currents were recorded at indicated holding potentials in symmetrical 250 mM KCl with 2–20 μ M Ca^{2+} and 1 mM ATP in the *cis* chamber as downward (left) or upward (right) inflections from the closed state (*c*–). (B–D) After addition of 500 nM ryanodine (B) and in subsequent presence of 500 nM *cis* (C) or 2 μ M *trans* neomycin (D).

(SR luminal) chamber, neomycin blocked wild-type RyR1 and D⁴⁹³⁸N at negative but not positive holding potentials (Figs. 3 and 4, and Fig. 6 B).

The opposite results were obtained for RyR1-D⁴⁸⁹⁹Q (Fig. 5). Neomycin induced a partial concentration- and voltage-dependent block from the cytosolic side, but failed to do so from the SR luminal side. Voltage dependence of neomycin blocks on wild-type and mutant channels are summarized in Table 2. The results indicate that D⁴⁹³⁸ and D⁴⁹⁴⁵ are located in the cytosolic vestibule of RyR1. They further confirm that the negatively charged amino acid residues D⁴⁸⁹⁹ and E⁴⁹⁰⁰ are located on the luminal side of the selectivity filter.

Kinetics of neomycin interaction with ryanodine-modified RyR1 mutant channels

The interactions of neomycin with the ryanodine-modified RyR channels were analyzed by determining the concentration dependence of the probability of substate occurrence (P_s). The dependence of P_s on the concentration of *cis* neomycin at +70 mV and *trans* neomycin at a holding potential of –60 mV is shown for wild-type RyR1 and D⁴⁹³⁸N in Fig. 7 A. The data were fit with Eq. 2 and for wild-type yielded an apparent K_{50} of 0.1 and 0.8 μ M for *cis* and *trans* neomycin, respectively (Table 2).

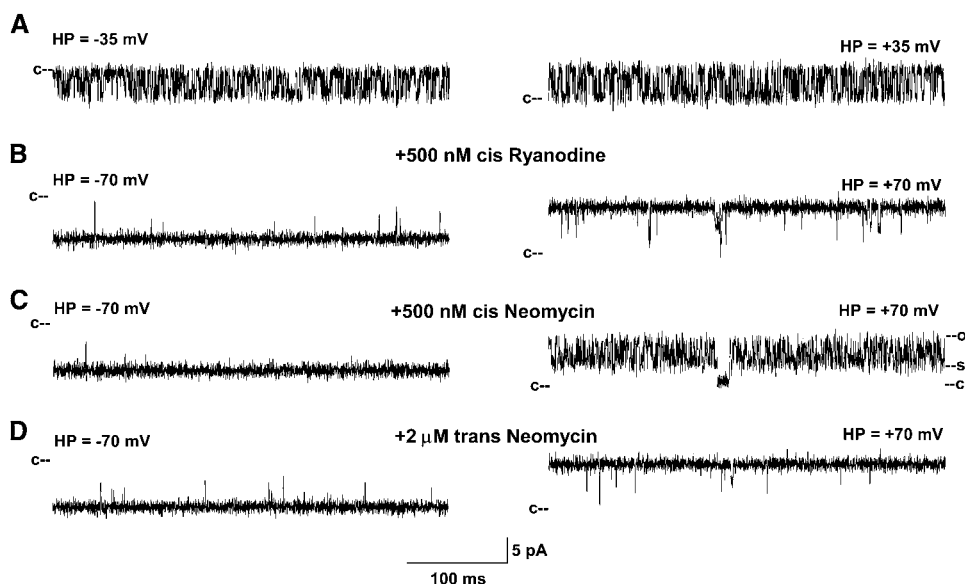


FIGURE 5 Single channel recordings of RyR1-D⁴⁸⁹⁹Q. (A) Single channel currents were recorded at indicated holding potentials in symmetrical 250 mM KCl with 2–20 μ M Ca^{2+} and 1 mM ATP in the *cis* chamber as downward (left) or upward (right) inflections from the closed state (*c*–). (B–D) After addition of 500 nM ryanodine (B) and in subsequent presence of 500 nM *cis* (C) or 2 μ M *trans* neomycin (D).

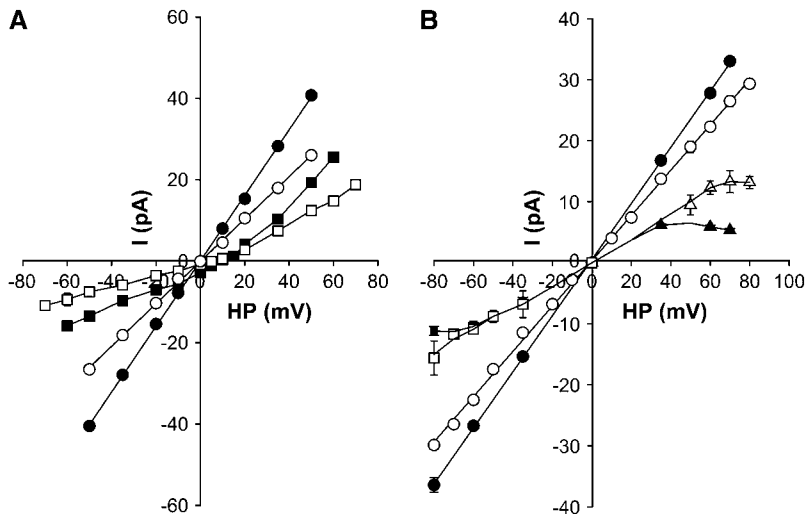


FIGURE 6 Current-voltage relationships of wild-type RyR1 and RyR1-D⁴⁹³⁸N. (A) Representative current-voltage relationships in 250 mM symmetrical KCl (●, wild-type; ○, D⁴⁹³⁸N) and with the subsequent addition of 10 mM *trans* Ca²⁺ (■, wild-type; □, D⁴⁹³⁸N). (B) Representative current-voltage relationships in 250 mM symmetrical KCl of ryanodine-modified channels (●, wild-type; ○, D⁴⁹³⁸N) and substate current-voltage relationships after the subsequent addition of 500 nM *cis* (▲, wild-type; △, D⁴⁹³⁸N) or *trans* (■, wild-type; □, D⁴⁹³⁸N) neomycin. Note, not shown is the absence of *cis* and *trans* neomycin block at negative and positive holding potentials, respectively.

Removal of negative charges from D⁴⁹³⁸N attenuated neomycin block to a greater extent from the *cis* than from the *trans* side. For D⁴⁹³⁸N, the averaged K_{50} of *cis* neomycin block was increased by >100-fold compared to wild-type. A ninefold increase in K_{50} of *cis* neomycin block was calculated for D⁴⁹⁴⁵N (Table 2). No changes in apparent affinity of neomycin block from the *cis* side were observed for RyR1-E⁴⁹⁵⁵Q. In comparison, charge neutralization of RyR1-D⁴⁸⁹⁹ and -E⁴⁹⁰⁰ predominantly affected neomycin block from the *trans* side. A blocking action of neomycin from the *trans* side was not observed for D⁴⁸⁹⁹Q and E⁴⁹⁰⁰N. Interestingly, charge neutralization also affected K_{50} of neomycin block of D⁴⁹³⁸N, D⁴⁹⁴⁵N, and E⁴⁹⁵⁵Q from the

trans side and block of D⁴⁸⁹⁹Q and E⁴⁹⁰⁰N from the *cis* side (Table 2).

We also determined the voltage and concentration dependence of association and dissociation rates of neomycin block. Consistent with the above data, the on rate of *cis* neomycin block increased linearly as a function of neomycin concentration at +70 mV, yielding an association rate constant (k'_{on}) of $5.2 \text{ s}^{-1} \mu\text{M}^{-1}$ for wild-type and a greatly reduced association rate constant of $0.1 \text{ s}^{-1} \mu\text{M}^{-1}$ for D⁴⁹³⁸N (Fig. 7 B, Table 2).

The dissociation rates of neomycin decreased as the concentration of neomycin in the *cis* and *trans* chambers was increased to 1 μM (0.5 μM for wild-type) and 2 μM ,

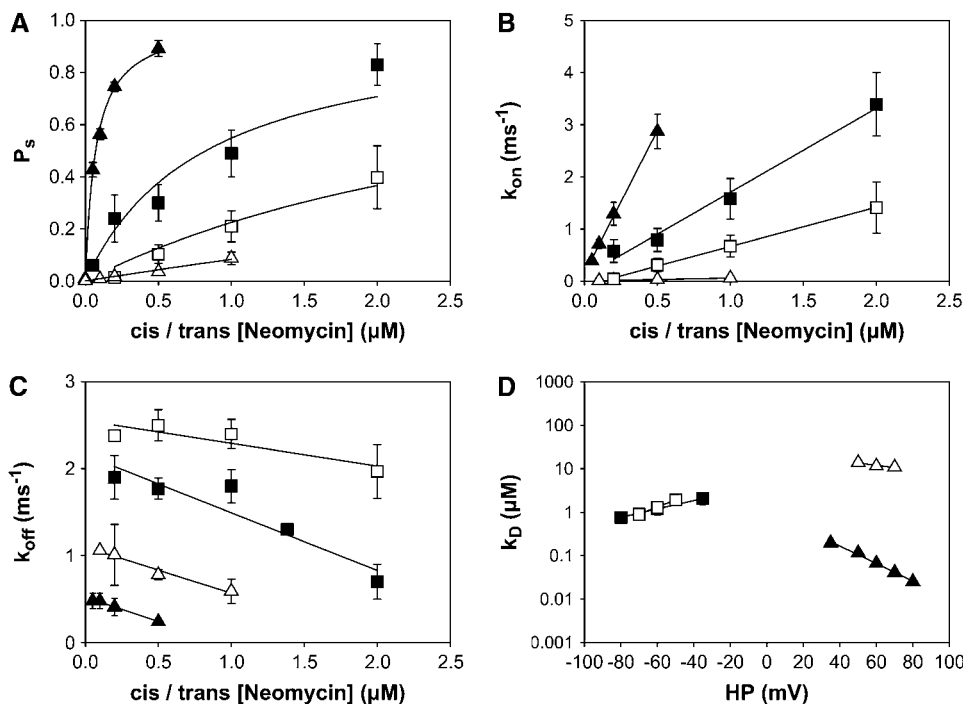


FIGURE 7 Kinetic parameters of neomycin block on wild-type RyR1 and RyR1-D⁴⁹³⁸N. (A) P_s dependence of wild-type and D⁴⁹³⁸N on *cis* (at +70 mV) and *trans* (at -60 mV) neomycin concentration. The lines represent the fits to the data using Eq. 2. (B and C) Dependence of association (B) and dissociation (C) rates of wild-type and D⁴⁹³⁸N on *cis* (at +70 mV) and *trans* (at -60 mV) neomycin concentration. k_{on} and k_{off} were obtained using Eqs. 3 and 4. (D) Relationship between K_D and holding potential of *cis* and *trans* neomycin block on wild-type and D⁴⁹³⁸N. Neomycin concentrations were 0.5 μM for wild-type and 1 μM for D⁴⁹³⁸N. Lines represent the fits obtained using Eq. 6. (A-D) *cis* neomycin (▲, wild-type; △, D⁴⁹³⁸N) and *trans* neomycin (■, wild-type; □, D⁴⁹³⁸N). Data were obtained from recordings similar to those shown for wild-type in Fig. 3, C and D, and for D⁴⁹³⁸N in Fig. 4, C and D. Each point represents the mean \pm SE of 4–7 experiments.

respectively (Fig. 7 C). The reasons for these decreases are unclear. Dependence of k_{off} on neomycin concentration was also observed for the native cardiac RyR2 (9). In contrast, ryanodine-modified RyR2 channels did not show neomycin dependence at low concentrations (0–200 nM) (10). In this study, k_{off} in Table 2 was determined at 0.1 μM *cis* and 0.5 μM *trans* neomycin, i.e., where k_{off} was largely independent on neomycin concentration (Fig. 7 C). The ratio of the rate constants, $k_{\text{off}}/k_{\text{on}}$, for wild-type and D⁴⁹³⁸N in the presence of *cis* neomycin yielded K_D of 0.1 and 8 μM . Respective ratios in the presence of *trans* neomycin were 0.9 and 3.3. The four ratios were in reasonable agreement with dissociation equilibrium constants (K_{50}) obtained using probability function curves of substate occurrence (P_s).

The voltage dependence of the rate constants was analyzed by the Boltzmann equation (Eq. 6). As shown in Fig. 7 D, K_D of *cis* neomycin block on wild-type RyR1 and D⁴⁹³⁸N decreased as the holding potential was made more positive. K_D of *trans* neomycin block decreased as the holding potential became more negative. The solid lines drawn through the points were obtained by linear regression, and the values of total effective gating charges (z_{total}) obtained from the slopes of these lines for *cis* neomycin were 1.1 for wild-type and 0.4 for D⁴⁹³⁸N. Similar z_{total} values were obtained for *trans* neomycin (−0.6 and −0.7 for wild-type and D⁴⁹³⁸N, respectively).

The kinetic parameters of neomycin block from the *cis* and *trans* sides for wild-type and mutants are summarized in Table 2. For D⁴⁹³⁸N, an ~50-fold decrease in the association rate constant was primarily responsible for the reduced affinity of neomycin binding from the *cis* side. z_{total} was 0.4 compared to 1.1 for wild-type, suggesting a change in the location of the neomycin binding site and/or the voltage drop when the charge on D⁴⁹³⁸ is neutralized. For D⁴⁹⁴⁵N, a 2.4-fold decrease in k_{on} and a 4-fold increase in k_{off} yielded a nearly 10-fold increase in the equilibrium dissociation constant compared to wild-type without a change in z_{total} . Significant differences in the rate constants were also calculated for D⁴⁹³⁸N, D⁴⁹⁴⁵N, and E⁴⁹⁵⁵Q from the *trans* side and for D⁴⁸⁹⁹Q from the *cis* side. However, these differed by <3-fold compared to wild-type. Collectively, the data are consistent with the notion that neomycin blocks the skeletal muscle RyR by binding with high affinity to cytosolic (D⁴⁹³⁸) and luminal (D⁴⁸⁹⁹, E⁴⁹⁰⁰) pore sites.

DISCUSSION

This study identified by mutagenesis negatively charged residues that affect K⁺ ion permeation (D⁴⁹³⁸, D⁴⁹⁴⁵) and Ca²⁺ ion selectivity (D⁴⁹³⁸). No changes in K⁺ ion permeation and Ca²⁺ selectivity were observed for two other RyR1 mutants (E⁴⁹⁵²Q, E⁴⁹⁵⁵Q). These four mutants (D⁴⁹³⁸N, D⁴⁹⁴⁵N, E⁴⁹⁵²Q, E⁴⁹⁵⁵Q) maintained caffeine-induced Ca²⁺ release, [³H]ryanodine binding, and regulation by Ca²⁺ in [³H]ryanodine binding and single channel measurements,

indicating no major alterations in protein structure. In a previous study, we showed that two conserved negatively charged amino acid residues (D⁴⁸⁹⁹, E⁴⁹⁰⁰) immediately after the selectivity filter motif GGGIG play a critical role in RyR1 ion permeation and selectivity (21). Charge neutralization of D⁴⁸⁹⁹, and to a lesser extent of E⁴⁹⁰⁰, reduced K⁺ ion conductance and selectivity for Ca²⁺ compared to K⁺.

We further showed that charge neutralization of D⁴⁹³⁸ (and to a lesser extent D⁴⁹⁴⁵) attenuated the block of neomycin from the cytosolic side, whereas charge neutralization of D⁴⁸⁹⁹ and E⁴⁹⁰⁰ removed the block from the luminal side. The results suggest the presence of rings of negative charges in the luminal (D⁴⁸⁹⁹ and E⁴⁹⁰⁰) and cytosolic (D⁴⁹³⁸ and D⁴⁹⁴⁵) vestibules that are required for maintaining the high rates of RyR1 ion fluxes. They further suggest that the luminal D⁴⁸⁹⁹ and E⁴⁹⁰⁰ and cytosolic D⁴⁹³⁸ loci represent major cation-blocking sites in the tetrameric RyRs.

Previous studies have indicated that mutations in the final membrane domain affect RyR channel function. Naturally occurring mutations that give rise to two skeletal muscle-associated diseases, malignant hyperthermia and central core disease, are clustered in three regions of RyR1, one of which is the pore-forming region of RyR1 (31). A RyR1-G⁴⁹⁴¹ mutation was detected in a patient whose muscle biopsy exhibited a sustained increase in muscle tension at a low halothane concentration (32). In another patient, deletion of two amino acid residues (V⁴⁹²⁶, I⁴⁹²⁷) caused central core disease (33). The deletion abolished [³H]ryanodine binding and Ca²⁺ responsiveness, and in single channel measurements exhibited a reduced K⁺ conductance and Ca²⁺ selectivity, indicating major alterations in protein conformation and pore structure (34). Wang et al. (35,36) used alanine and glycine scanning mutagenesis to show that the final membrane domain is an important determinant of [³H]ryanodine binding and channel activity. In another study, two RyR1-D⁴⁹³⁸ mutants (D⁴⁹³⁸N, D⁴⁹³⁸E) maintained caffeine-induced Ca²⁺ release and [³H]ryanodine binding (37). This work extends the above studies by showing that mutagenesis of RyR1-D⁴⁹³⁸ and D⁴⁹⁴⁵ to asparagine modified RyR1 ion fluxes.

We used the large polycation neomycin to probe the location of negatively charged amino acid residues. In agreement with previous single channel measurements of the cardiac ryanodine receptor (9–11), neomycin induced partial blocking events when added to the luminal or cytosolic side of wild-type RyR1. To facilitate analysis of interaction of neomycin with our mutant channels, experiments were performed using ryanodine-modified channels. For all mutants, differences in blocking parameters were observed. The most significant effect of removing a negative charge was observed for D⁴⁸⁹⁹Q and E⁴⁹⁰⁰N, as addition of neomycin to the *trans* chamber failed to produce noticeable blocking events in the two mutant channels. Removal of negative charge from D⁴⁹³⁸ had a less severe effect. It greatly attenuated, but did not eliminate, neomycin block from the cytosolic side.

Charge neutralization primarily affected the blocking potency of *cis* neomycin by decreasing the on rate of neomycin binding to D⁴⁹³⁸N. The results suggest a lessening of attractive charge interactions between the polycation neomycin and channel regions of high negative charge density. Because of the proximity of four negative charges in the homotetrameric RyR, such regions are expected to be formed on the luminal (D⁴⁸⁹⁹ and E⁴⁹⁰⁰) and cytosolic (D⁴⁹³⁸) sides of the selectivity filter. Interestingly, charge neutralization of luminal D⁴⁸⁹⁹ influenced the magnitude and affinity of neomycin block from the *cis* side, and vice versa the cytosolic mutants D⁴⁹³⁸N, D⁴⁹⁴⁵N, and E⁴⁹⁵⁵Q affected neomycin block from the luminal side. The reason for this is unclear. One possibility we cannot rule out is that these mutations modified the structure of the pore.

Mutagenesis also resulted in a change of the voltage dependence of neomycin block in one mutant (D⁴⁹³⁸N). Total effective gating charges (z_{total}) of wild-type RyR1 for cytosolic and luminal neomycin were 1.1 and -0.6 , respectively. Dividing these numbers by the nominally four positive charges of neomycin yields an electrical distance of 0.27 from the cytosolic side and 0.15 from the luminal side for the location of the neomycin binding sites in the voltage drop of wild-type RyR1. However, these distances may be substantially underestimated because it is not known how many of the positive charges of neomycin interact with the voltage drop. z_{total} values comparable to wild-type indicate a similar relative location of cytosolic- and luminal neomycin-blocking sites in wild-type and mutant channels. One exception was RyR1-D⁴⁹³⁸N where a 2–3-fold reduction in z_{total} for *cis* neomycin suggests a major change in the relative location of the neomycin-blocking site, voltage drop, or both.

One model of the RyR pore structure that is consistent with our results is shown in Fig. 8. The model is based on the known structure of the K⁺ channels (26) and is in good agreement with two recent cryoelectron microscopy studies that determined the pore structure of the closed RyR1 ion channel at a resolution of 13.6 Å (38) and 9.6 Å (39), based on a Fourier shell cutoff criterion at 0.5. Both studies revealed a RyR1 pore structure similar to that of K⁺ channels. Structural elements visualized in the RyR1 transmembrane domain at 13.6 Å resolution included a shallow luminal vestibule, a constriction of the pore (the selectivity filter), an inner vestibule, four high-density rod-like structures that merged into a dense ring (termed ion gate), and a wide cytosolic vestibule (38). Ludtke et al. (39) resolved at the higher resolution five helices in the membrane-spanning region including a transmembrane helix and short helix both lining the pore. A kink in the transmembrane helix suggested the presence of a “gating hinge” glycine (G⁴⁹³⁴) as observed in K⁺ channels (26).

In the model shown in Fig. 8, we introduced a break near RyR1-D⁴⁹³⁸ in the helical structure of the putative inner helix of the RyR1 ion pore, based on secondary structure predictions. RyR1-D⁴⁹³⁸ is part of a conserved GLIDAF motif

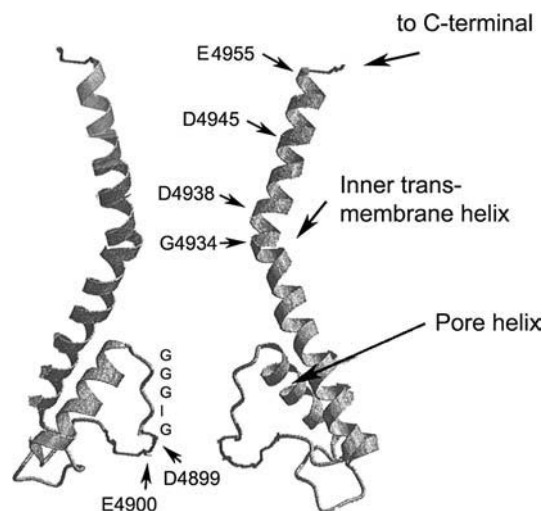


FIGURE 8 Hypothetical model for ion pore of RyR1. The RyR1 pore model, which was obtained by modifying by “hand” the published structure of K⁺ channels (24,26), shows the putative position of luminal amino acid residues D⁴⁸⁹⁹ and E⁴⁹⁰⁰ and cytosolic amino acid residues G⁴⁹³⁴, D⁴⁹³⁸, D⁴⁹⁴⁵, and E⁴⁹⁵⁵. The inner transmembrane helix in RyR1 is analogous to S6 in 6TM K⁺ channels (such as *Shaker* and *KvAP*) and M2 in 2TM K⁺ channels (such as *KcsA* and *Kir*). Note only two of the four pore-forming segments are shown.

in the RyRs and GVIIDTF motif in the related inositol receptors (IP₃Rs). Secondary structure predictions indicate a high helical probability for the putative inner helix and succeeding ~16 amino acid residues (Fig. 1 C). A decrease at RyR1-G⁴⁹³⁴ suggests a potential break in the helical structure of the inner helix and widening of the cytosolic vestibule, as has been found in K⁺ channels (26). In K⁺ channels, a “gating hinge glycine” residue bends the inner helix by ~30° and widens the cytosolic vestibule of the open channels. The presence of an enlarged RyR1 vestibule is consistent with our observation that acidic residues following RyR1-D⁴⁹³⁸ appear to have a lesser role in RyR1 ion permeation and selectivity. We also note that an extended cytosolic vestibule (38,39) can be expected to favor retention of neomycin in the conductance pathway at positive voltages and provides a likely explanation that neomycin blocks RyRs more effectively from the cytosolic than luminal side ((11), this study).

Two models have been recently described to account for the ion fluxes through RyRs. Molecular dynamic simulations identified several kinetically important residues that slowed the velocity of K⁺ and Ca²⁺ movement through the RyR2 pore (40). At variance with our experimental results ((21), this study), RyR2-D⁴⁸³¹ (equivalent to RyR1-D⁴⁸⁹⁹) and RyR2-D⁴⁸⁷⁰ (equivalent to RyR1-D⁴⁹³⁸) were not identified as major kinetic barriers that reduced the rates of ion translocation.

In a model of ion permeation through RyR, we have shown that the effects of neutralizing the negatively charged carboxyl oxygens of RyR1-D⁴⁸⁹⁹ and -E⁴⁹⁰⁰ are well

reproduced by a Poisson-Nernst-Planck/Density Functional Theory (PNP/DFT) model that describes the electrodiffusion of finite-sized ions down their chemical potential gradients (41). Interestingly, the model suggests that, in addition to the negatively charged luminal residues D⁴⁸⁹⁹ and E⁴⁹⁰⁰, some negative charges in the cytosolic atrium are critical for maintaining high rates of RyR ion flux and selectivity. Whether D⁴⁹³⁸ and D⁴⁹⁴⁵ can account for the negative charges in the cytosolic vestibule predicted by the PNP/DFT model remains to be determined.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

The authors thank Daniel Pasek and Kelly Evans for purifying and determining the activities of the RyR1 mutants.

Support by National Institutes of Health Grant AR18687 is gratefully acknowledged.

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