Ryanodine-Induced Structural Alterations in the RyR Channel Suggested by Neomycin Block

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ABSTRACT In Mead and Williams, (*Biophys. J.* 82:1953–1963, 2002) we have reported that neomycin is a potent partial blocker of single purified sheep cardiac SR calcium release channels. Neomycin is unusual in that it is capable of blocking when applied to either the cytosolic or the luminal face of the channel. Block at either aspect of the channel is both concentration- and voltage-dependent, but exhibits different blocking parameters. In this study we have investigated the actions of neomycin on ion handling in the ryanodine-modified channel. Neomycin is more effective at the cytosolic face, having a $K_b(0)$ value of 534.9 ± 35.17 nM compared with a $K_b(0)$ value of 971.5 ± 66.62 nM for the luminal face. The voltage dependence also differs at the two sites. Values of $z\delta$ for cytosolic and luminal neomycin are 1.09 ± 0.04 and −0.57 ± 0.03, respectively. The interaction of neomycin with the ryanodine-modified channel differs notably from that in the unmodified channel. Voltage-dependent relief of block is not observed after ryanodine modification, and the luminal blocking characteristics are altered. This suggests that ryanodine induces changes at the luminal mouth of the channel and may confer increased rigidity to the channel protein.

INTRODUCTION

In Mead and Williams (2002), we described interactions of the polyamine aminoglycoside antibiotic neomycin with sites at the cytosolic and luminal faces of the sheep cardiac muscle ryanodine receptor channel (RyR). These interactions produce reduced conductance events as the result of partial block of the open channel. The probability of occurrence of both cytosolic and luminal neomycin-induced partial blocking events was influenced by blocker concentration and transmembrane holding potential. The affinity of the luminal neomycin site is significantly greater than that of the cytosolic site. Neomycin-induced partial block of the ryanodine receptor channel is relieved at high transmembrane holding potentials as the result of blocker permeation of the channel.

The interaction of ryanodine and its derivatives (ryanoids) with RyR results in profound alterations in channel function: single-channel open probability (P_o) increases dramatically and single-channel current amplitude is reduced. Reduced rates of permeant cation translocation reflect changes in the relative permeability of ions and the affinity of the conduction pathway of the channel for some ions when ryanodine is bound (Lindsay et al., 1994). In addition, the binding of ryanoids to RyR alters the blocking characteristics of impermeant cations such as tetrabutylammonium (Tinker and Williams, 1993b), cocaine (Tsushima et al., 1996), and tetraethylammonium (Tanna et al., 2001) in RyR.

Neomycin and [^{3}H]-ryanodine have been shown to bind simultaneously and noncompetitively at different sites in the skeletal muscle RyR channel (Wang et al., 1996). In this communication we report experiments in which we have investigated the factors governing the interaction, and the functional consequences of the interaction, of neomycin with ryanodine-modified RyR channels. A comparison of the data arising from these studies with those obtained by monitoring the interaction of neomycin with RyR channels in the absence of ryanodine reveals novel information on both the mechanisms involved in channel block by neomycin and the alteration of channel function by ryanodine.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum (SR) membrane vesicles and purification of the ryanodine receptor

The preparation of sheep cardiac muscle SR vesicles and the subsequent solubilization and purification of the ryanodine receptor were carried out as described in Mead and Williams (2002).

Reconstitution of purified RyR into planar bilayers and channel modification by ryanodine

RyR channels were incorporated into phosphatidylethanolamine planar bilayers as described in the preceding communication. However, for this study, all single channels were modified with ryanodine before addition of neomycin. Ryanodine modification was accomplished by addition of 100–200 nM ryanodine to the *cis* chamber (cytosolic face of the channel). When modification had occurred (indicated by the occurrence of a characteristic reduced-conductance state), the chamber was perfused with 210 mM K^+ to remove unbound ryanodine.

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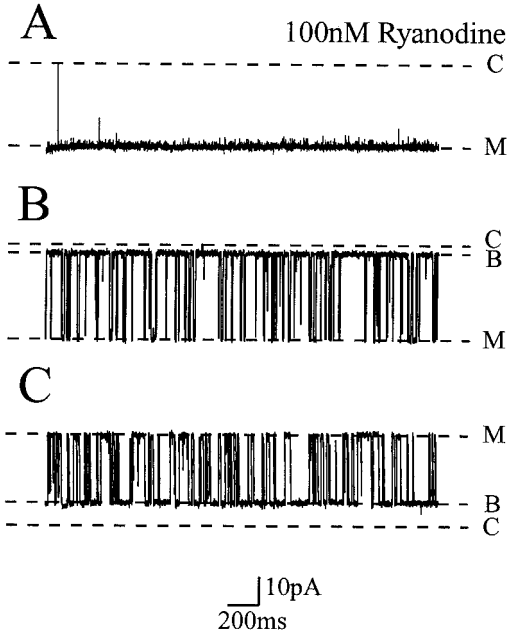

FIGURE 1 Representative channel recordings showing the effect of neomycin on a single purified ryanodine-modified ryanodine receptor channel. The upper control trace (*A*) was recorded at +60 mV in 210 mM K^+, which is the permeant ion solution used in all the experiments described in this report. The lower traces show the same channel after addition of 100 nM neomycin to the cytosolic (*B*) or luminal (*C*) face of the channel. Trace (*C*) was recorded at −60 mV. The dotted lines represent the closed channel level, denoted *C*, the subconductance level representing neomycin block, denoted *B*, and the modified open level, denoted *M*.

Data acquisition and analysis

Single-channel current fluctuations were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). For analysis, data were replayed, low-pass filtered with an 8-pole Bessel filter at 1 kHz, and digitized at 4 kHz using an AT-based computer system (Intracel, Cambridge, UK). As outlined above, ryanodine modification resulted in the opening of the channel to a subconductance state, with an effective open probability approaching 1.0 (Fig. 1). The subsequent addition of neomycin to either face of the ryanodine-modified channel resulted in rapid, clearly distinguishable blocking events. These blocking events were to a distinct level, a subconductance state, distinguishable from the normal closed level. We observed no increase in the occurrence of full closing events, suggesting that the subconductance state is the only indicator of block. The occurrence of block was assessed by monitoring channel open probability, which was determined by 50% threshold analysis with cursors set manually on the open and blocked levels. This analysis procedure was also used to ascertain mean dwell times in the open and blocked states. Current fluctuations to the closed level were omitted from the analysis, so all data relate only to neomycin-induced block of the open-modified RyR channel. Under the conditions used for analysis a dead time of 0.5 ms was determined. The impact of this was determined as described in Mead and Williams (2002). Distributions of dwell times in the open-modified and neomycin-induced subconductance states were adequately described by exponential distributions with a single component. Time constants derived from these distributions were not significantly different from mean dwell times determined from all monitored events. Data are presented as mean ± SEM. Linear and nonlinear regression analysis was carried out using GraphPad Prism.

Materials

The structure of neomycin is shown in Fig. 1 of Mead and Williams (2002). The net charge of neomycin at pH 7.4 is +4.4 (Haws et al., 1996). All solutions were prepared using de-ionized water produced by a Milli-Q water purification system. Ryanodine was obtained from Agrisystems International (Wind Gap, PA); [^{3}H]-ryanodine was obtained from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Bucks., UK); neomycin was obtained from Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK) and phosphatidylethanolamine was obtained from Avanti Polar Lipids (Alabaster, AL).

RESULTS

Initial observations

The first observation of note from our investigations is that, with potassium as the permeant ion, neomycin causes a partial block in the ryanodine-modified channel. The interaction of ryanodine with the high-affinity site on RyR modifies channel function. Single-channel conductance is reduced and open probability increases dramatically. The channel may occasionally close, but these closing events are rare and brief, so that open probability approximates to 1.

Under these conditions, block of potassium current by cytosolic neomycin is evident as fluctuations or blocking events to a current level distinct from the normal closed level, yielding a single subconductance state (Fig. 1). As noted in the Methods section, this allows analysis of block without interference from normal channel closings, as these are readily identifiable, and can be excluded from open probability measurement and dwell time analysis. Blocking events have durations in the millisecond range. As a result, individual events are clearly defined and block can be observed as a reduction in channel open probability.

The second important observation is that neomycin induces block when applied to either the cytosolic or the luminal face of the ryanodine-modified channel. Luminal and cytosolic block of the ryanodine-modified channel by neomycin are qualitatively similar; in both cases the polycation induces the occurrence of well-resolved reductions in current amplitude during channel openings; however, at equivalent holding potentials the amplitude of the blocked events differ. At a holding potential of +60 mV 100 nM cytosolic neomycin produces a blocked level that is ~10% of the modified open level (Fig. 1 *B*). At −60 mV the amplitude of the reduced-conductance state induced by the same concentration of luminal neomycin is ~30% of the modified open level (Fig. 1 *C*). In both cases, block only occurs when the holding potential across the membrane is such that the driving force favors the flow of both permeant and blocking cations into the channel; neomycin-induced subconductance states occur at positive holding potentials when the polycation is present at the cytosolic face of the channel and at negative holding potentials with luminal neomycin. In both cases the effects of neomycin are fully reversible on washout (not shown).

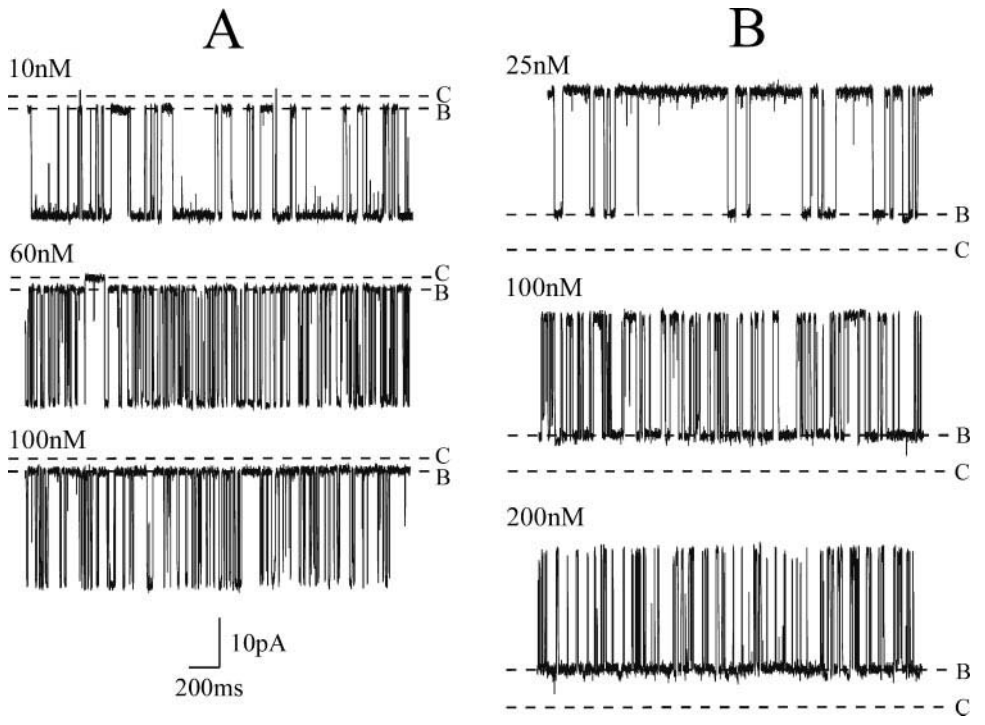

FIGURE 2 The effect of increasing neomycin concentration on open probability (P_o) of individual ryanodine-modified channels. Representative single-channel recordings of (*A*) cytosolic neomycin recorded at +60 mV and (*B*) luminal neomycin recorded at −80 mV. These traces show a reduction in P_o with increasing neomycin concentration from a control P_o of ~1 (see Fig. 1). There is a notable difference in the subconductance levels induced at either face, although these do not alter with increasing blocker concentration. *C*, closed; *B*, blocked level.

Given these initial observations, a simple open channel blocking scheme was assumed in which a single blocking molecule enters the conduction pathway of the ryanodine-modified channel to induce the observed subconductance states:

$$\text{modified-open} \underset{k_{off}}{\overset{k_{on}[n]}{\longleftrightarrow}} \text{subconductance*}$$

SCHEME 1

The asterisk indicates the amplitude of the partially blocked subconductance state is dependent upon the site of application of neomycin; k_{on} and k_{off} are, respectively, rate constants for the association of neomycin with and dissociation of neomycin from the modified-open RyR channel. In such a scheme the probability of occurrence of the subconductance state should be dependent upon the concentration of neomycin ([*n*]). In addition, if neomycin induces a partial block of K^+ translocation by interacting with sites within the conduction pathway of the channel, it is probable that either or both k_{on} and k_{off} will be influenced by transmembrane holding potential. Neomycin has access to the conduction pathway from either side of the channel and the amplitude of the resulting subconductance state differs depending on the route of entry. This scheme forms the basis for further studies and analysis of the mechanisms involved in blockade of the ryanodine-modified RyR channel by neomycin.

Concentration-dependence of neomycin block

At a fixed holding potential, increasing the concentration of neomycin applied at either face of the ryanodine-modified channel resulted in an increasing occurrence of blocking events. The parameters of the blocking interaction differ for cytosolic and luminal neomycin. The blocking events induced by various concentrations of neomycin, and the differences in block induced at the two sites, are clearly represented in the traces shown in Fig. 2. Irrespective of the side of application, the amplitude of the blocked subconductance state is independent of neomycin concentration. Variation in the occurrence of block with changing neomycin concentration was assessed as the reduction in open probability of single ryanodine-modified channels, and the data for both cytosolic and luminal block were described by a single site binding scheme of the form:

$$1 - P_o = B_{max} \cdot \frac{[\text{neomycin}]}{K_m + [\text{neomycin}]} \qquad (1)$$

Where $1 - P_o$ represents the probability of block occurring, B_{max} is the maximal degree of block, and K_m is the concen-

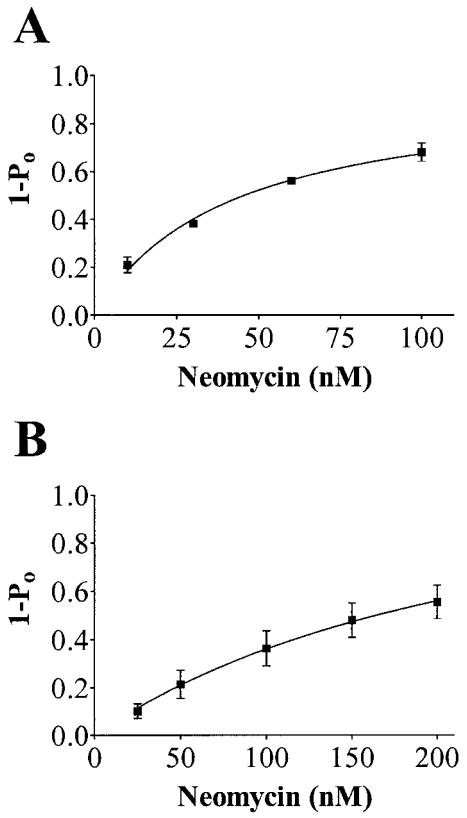

FIGURE 3 The relationship between increasing neomycin concentration and the probability of block ($1 - P_o$). This is determined by 50% threshold analysis, which is described in Materials and Methods. (*A*) Effect induced by cytosolic neomycin at a holding potential of +60 mV ($n = 4$); (*B*) luminal block at −80 mV ($n = 6$). The curves were generated by nonlinear regression, as described in the text. All values derived from the graphs are quoted in the text.

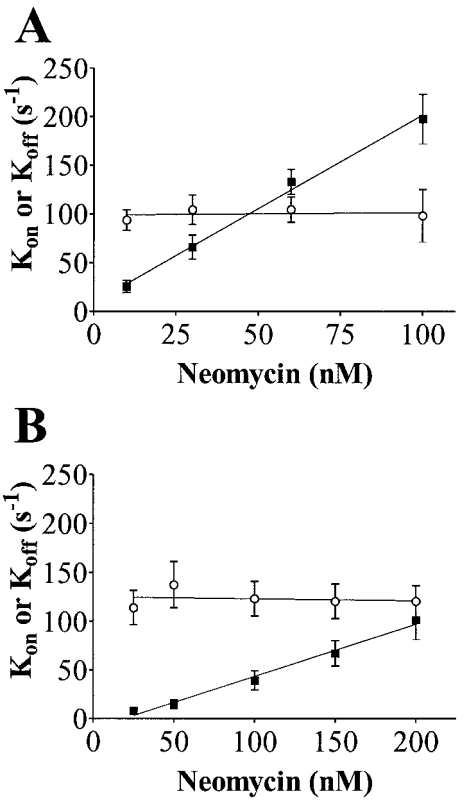

FIGURE 4 The relationship between increasing neomycin concentration and association (K_{on}, ■) and dissociation (K_{off}, ○) rates. Rates were determined as described in the text from data obtained at +60 mV for cytosolic block (*A*) and at −80 mV for luminal block (*B*). The lines drawn through all data were determined by linear regression. Data were obtained from a minimum of four experiments in all cases.

tration of neomycin at which half-maximal block occurs. Data accumulated from four channels with 10–100 nM cytosolic neomycin and four channels with 25–200 nM luminal neomycin are shown in Fig. 3, together with best-fit curves to Eq. 1 obtained by nonlinear regression. Analysis of the data derived from cytosolic blockade of the channel gives a B_{max} value of 0.96 ± 0.07 and a neomycin concentration producing 50% block (K_m) of 41.6 ± 7.3 nM at +60 mV. Equivalent calculations for luminal block at −80 mV yield the following values: B_{max} is calculated as 1.25 ± 0.1 and K_m as 246.6 ± 30.5 nM.

Information on the mechanisms underlying block of the ryanodine-modified channel by neomycin can be obtained by analysis of the mean dwell times in the modified-open and blocked states. Distributions of dwell times in both the modified-open and neomycin-induced subconductance states are monoexponential (see Materials and Methods). This is in keeping with the suggestion (Scheme 1) that the neomycin-induced subconductance states result from the interaction of a single molecule of neomycin with the modified-open channel. Under these circumstances, apparent rate constants for association of neomycin with the channel (k_{on}) can be calculated from the reciprocal of mean dwell times in the modified-open state, and apparent rate constants for dissociation of neomycin from the channel (k_{off}) can be calculated from the reciprocal of the mean dwell times in the blocked state. Fig. 4 shows the variations in k_{on} and k_{off} with changing concentrations of neomycin. Consistent with the proposals set out in Scheme 1, in the case of either cytosolic or luminal application of neomycin to the ryanodine-modified channel, the rate of association of neomycin with the channel varies linearly with polycation concentration, while the dissociation rate of the blocking cation is independent of concentration. Rates of dissociation of neomycin from the cytosolic and luminal blocking sites are similar, with mean values of 98.8 ± 9.0 s^{-1} and 125.0 ± 8.5 s^{-1}, respectively. However, rates of association of the polycation with the two sites are considerably different. Linear regression of the variation in rates of association of cytosolic and luminal neomycin with increasing polycation concentration (Fig. 4) yields values of 1.93 ± 0.1 nM^{-1} s^{-1} and 0.54 ± 0.03 nM^{-1} s^{-1}, respectively.

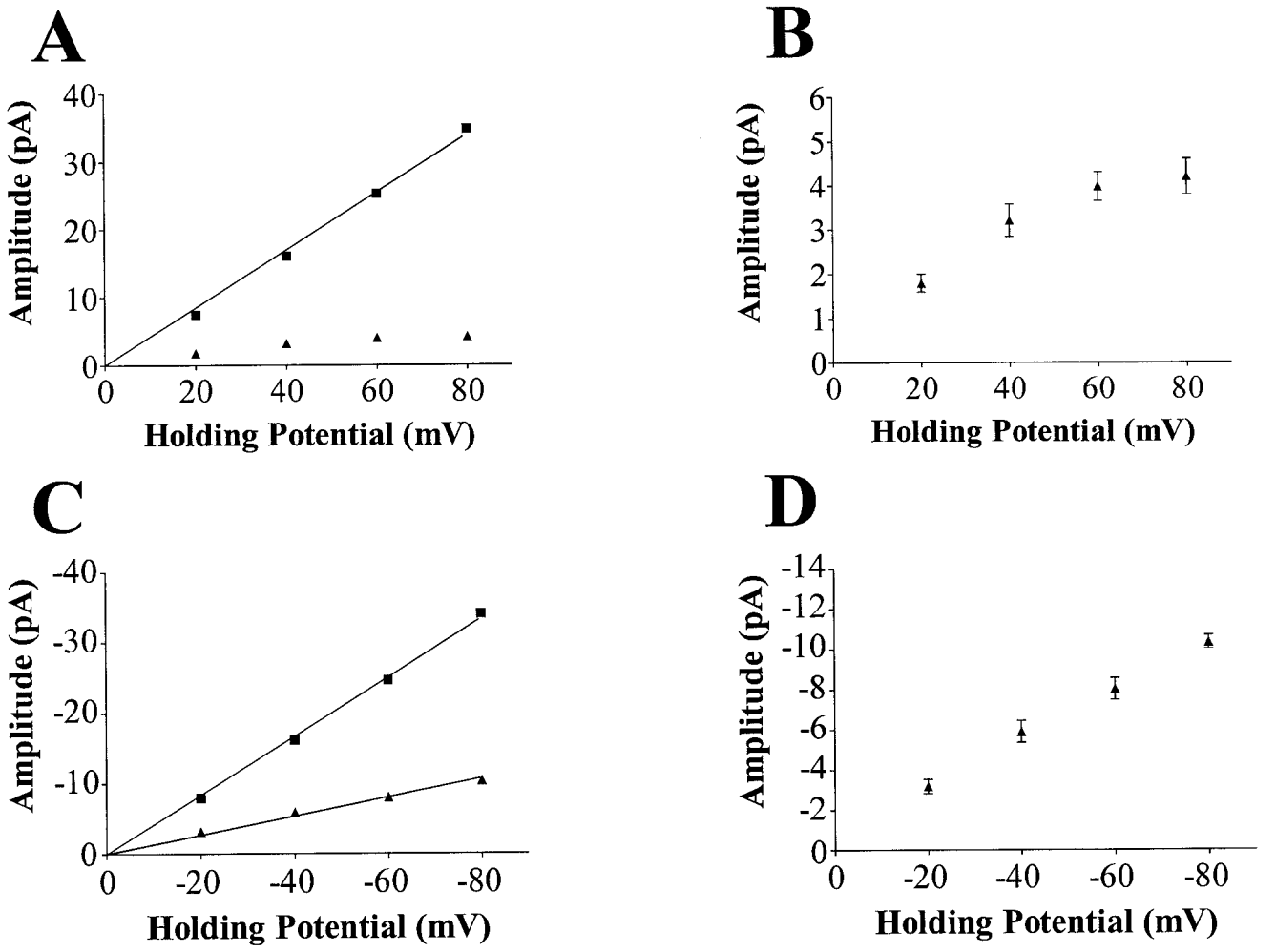

FIGURE 5 Current-voltage relationships of the ryanodine-modified open state and the neomycin-induced subconductance state. (*A*) Modified-open state (■) and neomycin-induced subconductance state current amplitude (▲) in the presence of 30 nM cytosolic neomycin. For clarity, variations in subconductance state current amplitude are shown on a expanded scale in (*B*). (*C*) Modified-open state (■) and neomycin-induced subconductance state current amplitude (▲) in the presence of 100 nM luminal neomycin. For clarity, variations in subconductance state current amplitude are shown on an expanded scale in (*D*).

Dissociation constants calculated from these values ($K_D = K_{off}/K_{on}$) are 51.2 nM for cytosolic neomycin and 232.2 nM for luminal neomycin, and are in good agreement with the K_D values determined from the variation in probability of occurrence of block with varying neomycin concentration (Eq. 1).

Effects of varying holding potential on neomycin block

As noted above, at holding potentials of ±60 mV the amplitude of the reduced conductance state induced by cytosolic neomycin differs from that induced by the same concentration of luminal neomycin. Although the amplitude of the neomycin-induced reduced conductance states is independent of neomycin concentration, we do observe some variations with changing holding potential (Fig. 5). As is the case with RyR in the absence of ryanodine, the amplitude of the reduced conductance state induced by the addition of neomycin to the cytosolic face of the ryanodine-modified channel varies only slightly as holding potential is increased from +20 to +80 mV (unitary conductance of the neomycin-induced subconductance state is voltage-dependent). In contrast, the amplitude of the reduced conductance state induced by the addition of neomycin to the luminal face of the ryanodine-modified channel varies linearly with holding potential, maintaining an amplitude of ~30% of the modified open level (unitary conductance of the neomycin-induced subconductance state is voltage-independent).

The probability of occurrence of both cytosolic and luminal neomycin-induced blocking events of the ryanodine-modified RyR channel is dependent upon holding potential. Fig. 6 shows the marked difference in proba-

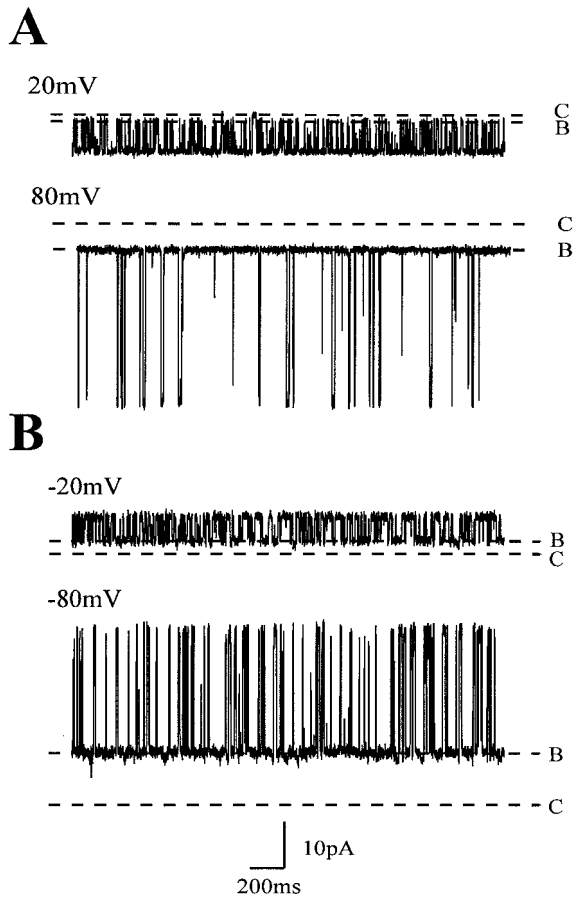

FIGURE 6 The influence of increasing holding potential on neomycin-induced block. The figure shows representative single-channel recordings in the presence of 100 nM cytosolic neomycin (*A*) or 200 nM luminal neomycin (*B*), showing the effect of varying holding potentials. Channel closed level is denoted by *C*, blocked level by *B*.

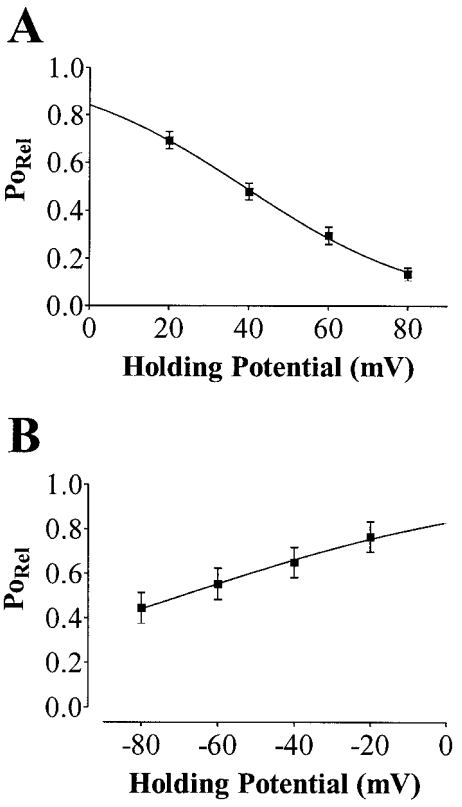

FIGURE 7 Graphs representing the relationship between various holding potentials and relative P_o in the presence of 100 nM cytosolic neomycin (*A*) or 200 nM luminal neomycin (*B*). The curves are nonlinear regression lines derived from Woodhull analysis of data obtained from a minimum of four experiments.

bility of occurrence of neomycin-induced blocking events at a fixed concentration of neomycin at holding potentials of ±20 and ±80 mV. For both cytosolic and luminal neomycin the probability of occurrence of block is greater at the higher holding potential. The relationships between channel open probability in the presence of neomycin ($P_{o_{rel}}$) and holding potential for four channels in the presence of 100 nM cytosolic neomycin (*A*) and four channels in the presence of 200 nM luminal neomycin (*B*) are shown in Fig. 7. The variation in the occurrence of cytosolic and luminal neomycin-induced block of the ryanodine-modified RyR channel with holding potential can be described by the Woodhull relationship (Woodhull, 1973), in which it is envisaged that the blocking cation interacts with a site located within the voltage drop across the channel. The blocking cation has access to the site from only one side of the channel. Under these circumstances the relationship between channel open probability, defined as the relative open probability ($P_{o_{rel}}$) and holding potential is given by:

$$P_{o_{rel}} = \left[1 + \frac{[\text{neomycin}]}{K_b(0)} \cdot \exp(z\delta \cdot FV/RT)\right]^{-1} \quad (2)$$

Where $K_b(0)$ represents the dissociation constant at 0 mV and $z\delta$ is the effective valence, which is the product of the valence of the blocking ion (z) and the fraction of the voltage drop across the channel sensed by the blocking cation (δ). F, R, and T have their usual meanings, and RT/F is 25.2 mV at 20°C. The values of $K_b(0)$ and $z\delta$ calculated for block by cytosolic neomycin are 534.9 ± 35.17 nM and 1.09 ± 0.035, respectively. The equivalent values for these parameters for luminal block are 971.5 ± 66.62 nM and −0.57 ± 0.03. A comparison of the blocking parameters obtained with cytosolic and luminal neomycin indicates that transmembrane holding potential has a more marked influence on the interaction of neomycin with the cytosolic site of interaction and that the affinity of the cytosolic site of interaction is greater than that of the luminal site. As is the case with block of the RyR channel by neomycin in the absence of ryanodine reported in Mead and Williams (2002)

the distribution of charge across the molecule makes it impossible to define a specific electrical distance (δ) for the site of interaction of the polycation within the voltage drop across the ryanodine-modified channel.

The observed variation in the probability of occurrence of neomycin-induced blocking events in the ryanodine-modified RyR channel with changing holding potential reflects voltage-dependent variations in rates of association of the blocking cation with and dissociation of the blocking cation from the channel. We have investigated the influence of voltage on rates of neomycin association and dissociation by monitoring K_{on} (the reciprocal of the mean time in the modified-open state) and K_{off} (the reciprocal of the mean time in the blocked state) at holding potentials between ± 20 and ± 80 mV. Variations in rates of association and dissociation of neomycin with and from the ryanodine-modified RyR channel can be described by the Boltzmann relationship:

$$K_{on}(V) = K_{on}(0) \cdot \exp[z_{on} \cdot (FV/RT)] \quad (3)$$

and

$$K_{off}(V) = K_{off}(0) \cdot \exp[-z_{off} \cdot (FV/RT)] \quad (4)$$

Where $K(V)$ and $K(0)$ refer to the rate constants at a given holding potential and 0 mV, respectively, and z is the valence of the respective reaction; z and $K(0)$ can be determined from the slope and intercept of plots of ln K_{on} or ln K_{off} against voltage. Such plots for the block of the ryanodine-modified RyR channel by cytosolic and luminal neomycin are shown in Fig. 8. An inspection of the relationships in Fig. 8 reveals that with either cytosolic or luminal neomycin the bulk of the variation in the probability of occurrence of block of the ryanodine-modified RyR channel induced by changing holding potential is derived from alterations in the rate of dissociation of the bound blocking cation from the channel. The rate of association of neomycin with either the cytosolic or luminal sites on the ryanodine-modified channel is effectively independent of voltage. The overall voltage dependence of each reaction (z_{total}) can be derived from the sum of z_{on} and z_{off}. For cytosolic neomycin z_{on} is 0.17, z_{off} is 1.00, and z_{total} is 1.17. For luminal neomycin z_{on} is -0.11, z_{off} is -0.65 and z_{total} is -0.76.

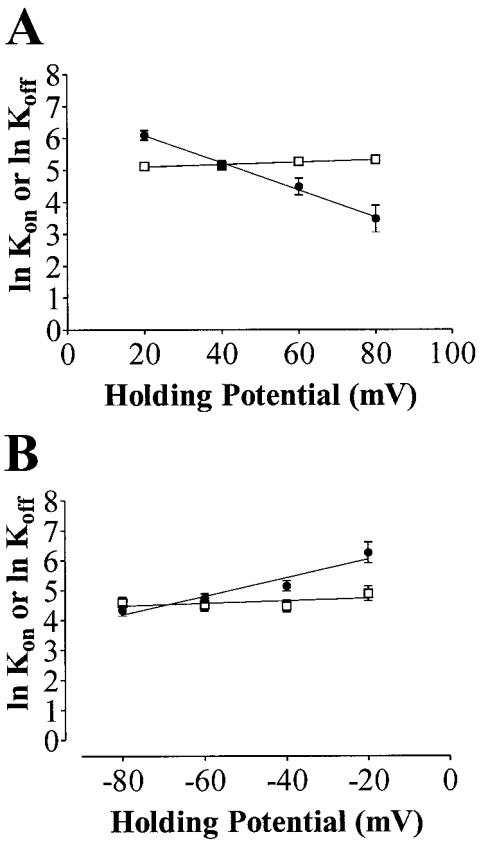

FIGURE 8 The relationship between holding potential and association (K_{on}, □) and dissociation (K_{off}, ●) rates for 100 nM cytosolic block (*A*) and 200 nM luminal neomycin (*B*). Rates were calculated as described in the text. All data derived from four or more experiments. The lines are obtained by linear regression as described in the text.

DISCUSSION

In Mead and Williams (2002), we demonstrated that the aminoglycoside antibiotic neomycin interacts with sites at both the cytosolic and luminal sides of the open RyR channel to induce the occurrence of reduced-conductance states. The probability of occurrence of the partial blocking events induced by either cytosolic or luminal neomycin was influenced by both polycation concentration and transmembrane holding potential. At high holding potentials neomycin block of RyR is relieved by permeation of the blocking cation.

The observations presented in this communication demonstrate that the interaction of ryanodine with the high-affinity binding site on the RyR channel results in what at first sight appear to be only minor alterations in the way in which neomycin interacts with, and blocks, the channel. Following modification of channel function by ryanodine, neomycin continues to be an effective blocking agent from both sides of the RyR channel. As in the unmodified RyR channel, blocking events are manifest as transitions from the open conductance level to a clearly resolved subconductance level, and the amplitude of the events induced by luminal neomycin is greater than that of those induced by cytosolic neomycin.

However, a closer inspection of the blocking behavior of neomycin in unmodified and ryanodine-modified RyR channels reveals some important quantitative differences that provide information on both the mechanisms involved in the block of RyR by neomycin and the consequences of the binding of ryanodine to the RyR channel. Neomycin

TABLE 1 Comparison of neomycin-blocking parameters in RyR in the absence of ryanodine and following ryanodine modification

	Cytosolic (Modified)	Cytosolic (Unmodified)	Luminal (Modified)	Luminal (Unmodified)
$z\delta$*	1.09 ± 0.04	1.05 ± 0.13	−0.57 ± 0.03	−0.66 ± 0.06
$K_b(0)$ (nM)*	534.90 ± 35.17	589.70 ± 184	971.50 ± 66.62	210.20 ± 22.80
K_{on} (s^{-1} nM^{-1})†	1.93 ± 0.10	2.23 ± 0.09	0.54 ± 0.03	2.51 ± 0.56
K_{off} (s^{-1})†	98.80 ± 9.00	129.15	125.40 ± 8.54	98.19 ± 8.59
K_D (nM)†	51.20	57.99	232.20	39.18

*$z\delta$ and $K_b(0)$ are calculated from the Woodhull fit of the data assessing the voltage dependence of neomycin block (see Fig. 9).
†K_{on} and K_{off} are calculated from assessment of varying neomycin concentration. K_D is calculated as K_{off}/K_{on}.

blocking parameters in unmodified and ryanodine-modified RyR channels are summarized in Table 1.

A comparison of the interactions of cytosolic neomycin with unmodified and ryanodine-modified RyR channels

The influence of changing neomycin concentration

We observe no significant difference in the blocking parameters derived for unmodified and ryanodine-modified channels in investigations involving alterations in cytosolic neomycin concentration. Concentrations of neomycin required to produce 50% block in unmodified and ryanodine-modified channels are identical, as are variations in rates of neomycin association and dissociation with changing neomycin concentration (Table 1). These investigations indicate that, at a fixed holding potential, the interaction of ryanodine with RyR produces no significant alteration in the interaction of the polycation with RyR from the cytosolic side of the channel.

The influence of transmembrane holding potential

We have used the method of Woodhull (1973) to analyze the variations in open probability with changing holding potential in the presence of cytosolic neomycin for both the unmodified and ryanodine-modified open RyR channels. The blocking parameters derived from these analyses (Table 1) indicate that modification of RyR channel function by ryanodine has no significant influence on the voltage dependence of the interaction of cytosolic neomycin ($z\delta$) or the affinity of the site ($K_b(0)$) at low holding potentials. In fact, the relationships between $P_{o_{rel}}$ and holding potential are indistinguishable within the +20 to +50 mV range for the two forms of the RyR channel (Fig. 9). However, at more positive potentials we observe a marked difference in the degree of block produced by cytosolic neomycin. In the unmodified open RyR channel, open probability increases and the relationship deviates markedly from the predictions of the Woodhull scheme. We have interpreted this behavior as relief of block as the channel becomes permeable to neomycin (Mead and Williams, 2002). No deviation from the predictions of the Woodhull model are seen at high positive holding potentials in the ryanodine-modified channel.

Consistent with these observations, variations in rates of neomycin association with changing voltage occur over a comparable range in unmodified and ryanodine-modified channels, and whereas in the unmodified channel we see increased rates of neomycin dissociation at high positive holding potentials, indicating that neomycin is permeant at these potentials, there is no increase in the rate of neomycin dissociation at comparable potentials in the ryanodine-modified channel. It would appear that the interaction of ryanodine with the RyR channel has little or no influence on the blocking characteristics of cytosolic neomycin at low transmembrane holding potentials; however, ryanodine modification of the channel prevents neomycin permeation at higher potentials.

The functional consequence of the interaction of cytosolic neomycin with both the unmodified and ryanodine-

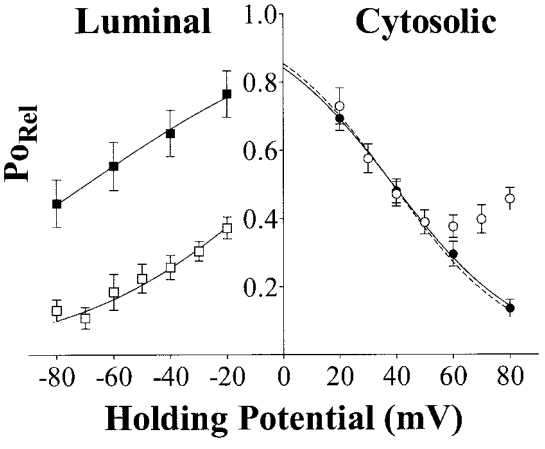

FIGURE 9 The relationship between holding potential and relative P_o for RyR channels in the presence of cytosolic (○) or luminal (□) neomycin. Open symbols represent data for 100 nM neomycin applied to either side of the unmodified channel, closed symbols represent data for 100 nM cytosolic or 200 nM luminal neomycin applied to the ryanodine-modified channel. Lines are nonlinear regression derived from the Woodhull analysis. The Woodhull fit (*dotted line*) obtained for cytosolic neomycin block of the unmodified channel was calculated using parameters obtained from a fit to data in the voltage range 20–50 mV.

modified RyR channels is a reduction in the rate of permeant ion translocation. The relationships between the conductance of the neomycin-induced states and holding potential in the unmodified and ryanodine-modified channels are similar; in both cases unitary conductance of the neomycin-induced subconductance state is reduced with increasing holding potential. This observation indicates that the mechanism underlying the reduction of potassium translocation in both forms of the channel (see Discussion in Mead and Williams, 2002) is similar and is consistent with other lines of evidence that suggest that the modification of RyR channel function by ryanodine has little effect on the blocking characteristics of cytosolic neomycin.

A comparison of the interactions of luminal neomycin with unmodified and ryanodine-modified RyR channels

The influence of changing neomycin concentration

The concentrations of luminal neomycin required to produce 50% block in unmodified and ryanodine-modified channels are very different. Ryanodine-modification of RyR decreases the affinity of the luminal neomycin site dramatically. An inspection of the variation in rates of association and dissociation of luminal neomycin with changing concentration of the polycation reveals that the alteration in affinity following the interaction of ryanodine results from a marked decrease in the rate of association of neomycin with the luminal site (Table 1).

The influence of transmembrane holding potential

In agreement with the conclusion reached in the preceding paragraph, a comparison of the blocking parameters obtained with luminal neomycin in unmodified and ryanodine-modified channels at a range of holding potentials demonstrates that the affinity of the luminal neomycin site is decreased markedly following the modification of channel function by ryanodine. The dissociation constant for luminal neomycin at 0 mV ($K_b(0)$) is approximately fivefold higher following ryanodine modification (Table 1). A comparison of association and dissociation rates of luminal neomycin over a full range of holding potentials reveals that the observed alteration in affinity reflects an approximate eightfold decrease in the rates of neomycin association with RyR following the binding of ryanodine to the channel (mean K_{on} in the absence of ryanodine is 4.45 s^{-1} nM^{-1}; mean K_{on} following ryanodine modification is 0.55 s^{-1} nM^{-1}).

Although the affinity of the luminal neomycin binding site is decreased by ryanodine-modification, interaction of the alkaloid with the channel has no significant influence on the voltage dependence of the reaction, whether measured as effective valence in the Woodhull scheme or from variations in rates of neomycin association and dissociation with voltage.

In Mead and Williams (2002), we presented evidence that indicated that the RyR channel might become permeable to luminal neomycin at holding potentials in excess of −70 mV. We see no deviation from the Woodhull scheme for block of the ryanodine-modified RyR channel by luminal neomycin at −80 mV, indicating that, following modification of channel function by ryanodine, any permeability to luminal neomycin is lost.

Ryanodine-modification of RyR also influences the functional consequence of the interaction of luminal neomycin with the channel. In the absence of ryanodine the unitary conductance of the neomycin-induced subconductance state decreased with increasing applied potential. In the ryanodine-modified channel the conductance of the reduced conductance state induced by luminal neomycin does not vary with holding potential.

What mechanisms are responsible for the observed ryanodine-induced alterations in neomycin block of RyR?

The interaction of ryanodine and derivatives of ryanodine with the RyR channel results in profound alterations in channel function; channel open probability increases dramatically and rates of translocation of inorganic divalent and monovalent cations are reduced. An increasing body of evidence suggests that the modification of RyR channel function by ryanodine reflects alterations in channel structure following the high-affinity binding of the alkaloid. Lindsay et al. (1994) demonstrated that the association of ryanodine with its high-affinity binding site on RyR produces changes in the relative permeability of ions and the affinity of the conduction pathway of the channel for some ions. It was proposed that these wide-ranging alterations in ion handling might reflect a ryanodine-induced alteration in the structure of the conduction pathway of the RyR channel.

In addition, the characteristics of interaction of impermeant cations, including local anesthetics, cocaine, and tetraalkylammoniums, are altered following ryanodine-modification of RyR. In the absence of ryanodine a variety of small tetraalkylammonium cations, local anesthetics, and organic cations block ion translocation in RyR by interacting with a site located ~90% into the voltage drop across the channel from the cytosolic face of the channel (Williams et al., 2001). In all cases these monovalent cations are only effective blockers from the cytosolic side of the channel. Tsushima et al. (1996) found that ryanodine-modification of RyR2 resulted in a dramatic reduction in the voltage- and concentration-dependence of the association rate of one of this group of blocking cations: cocaine. A similar reduction in the effective valence of block of another member of this group of cations, tetraethylammonium (TEA^+), was reported by Tanna et al. (2001). It was proposed that the

binding of ryanoids to the high-affinity ryanodine binding site on RyR induced conformational alterations in the channel that resulted in the relocation of the TEA^+ binding site within the voltage drop across the channel and alterations in the affinity of the channel for the blocking cation.

Large tetraalkylammonium cations such as tetrabutylammonium (TBA^+) and local anesthetics such as QX314 are believed to interact with sites located less deeply in the voltage drop across the RyR channel. In the absence of ryanodine these cations induce the occurrence of reduced-conductance events in RyR. The blockers are effective only from the cytosolic face of the channel, and the probability of occurrence of block is influenced by blocker concentration and transmembrane holding potential (Tinker et al., 1992; Tinker and Williams, 1993a). Xu et al. (1993) reported that ryanodine-modification produced a reduction in the effectiveness of QX314 as a cytosolic blocker of RyR1 that resulted largely from an approximate 35-fold reduction in the rate of association of the local anesthetic with the channel. Similar conclusions were reached by Tinker and Williams (1993b) who demonstrated that ryanodine-modification of RyR2 resulted in a threefold reduction in the affinity of the channel for TBA^+ that was accounted for predominantly by a marked reduction in the rate of association of the blocking cation with the channel. The voltage dependence of the interaction of TBA^+ with RyR2 was not affected by ryanodine-modification, indicating that the location of the blocker binding site within the voltage drop across the channel was not altered by ryanodine-modification. It was concluded that the alteration in the rate of association of TBA^+ and the concomitant reduction in affinity resulted from a ryanodine-induced reduction in the capture radius of the RyR2 conduction pathway.

The observations reported in this communication demonstrate that ryanodine-modification of the RyR2 alters the way in which neomycin interacts with the channel; however, the processes involved in these alterations differ greatly from those described above for other RyR blocking cations.

Ryanodine-modification has little or no influence on the partial block of RyR by cytosolic neomycin at low positive holding potentials. This contrasts markedly with the alteration in blocking parameters of the monovalent cation blockers described above, and probably indicates that the cytosolic site of neomycin interaction that results in the occurrence of partial block differs from those of the deep blockers, such as TEA^+, and/or the blockers that interact less deeply into the voltage drop, such as QX314.

The major impact of the putative ryanodine-induced structural rearrangements are seen at sites located at the luminal end of the voltage drop across the channel, such as the TEA^+ binding site ($\delta \sim 0.9$) (Tsushima et al., 1996; Tanna et al., 2001). The region of the RyR2 channel at which TEA^+ binds is almost certainly involved in the regulation of ion translocation and selection. Conformational alterations in this area, for example an alteration that resulted in an increased rigidity, could certainly contribute to the elimination of neomycin permeation of the channel following the interaction of ryanodine. An alternative possibility is that ryanodine, once bound to RyR, produces a steric barrier to neomycin translocation. The precise location of the high-affinity ryanodine binding site in RyR is unknown; however, circumstantial evidence supports the proposal that the site could be within the conduction pathway of the channel. Proteolytic cleavage of RyR (Callaway et al., 1994) indicates that [^{3}H]-ryanodine binds to the region of the molecule (the carboxyl-terminus) that contains components of the conduction pathway (Williams et al., 2001) and differences in the voltage dependence of interaction of charged and neutral derivatives of ryanodine with RyR have been interpreted as suggesting that the high-affinity binding site for these ryanoids is within the voltage drop across the channel (Tanna et al., 2000).

Neomycin is very unusual among blockers of RyR in that it is effective from both the cytosolic and luminal sides of the channel. As a consequence, neomycin provides us with a rare opportunity to investigate the luminal end of the RyR conduction pathway. The data presented in Mead and Williams (2002) demonstrated that, in the absence of ryanodine, neomycin is likely to interact with sites at the luminal extremity of the voltage drop across the RyR2 channel. Our observations reported in this communication demonstrate that this interaction is modified following the interaction of ryanodine with RyR2. Ryanodine-modification reduces the affinity of RyR2 for luminal neomycin, and this results from a decreased rate of association of the polycation with the channel. As discussed in the preceding communication, it is probable that long-range electrostatic interactions between the polycation and fixed charge on the channel molecule will be a major factor in the association of neomycin with the RyR channel. Alterations in the interaction of luminal neomycin following the modification of channel function by ryanodine may provide information on the mechanisms likely to underlie both the reduced affinity of the luminal site on RyR for neomycin and the abolition of neomycin permeation following ryanodine binding. The decreased rates of luminal neomycin association seen in the ryanodine-modified channel could result from a redistribution or masking of charge on RyR following a ryanodine-induced conformational change in the channel protein. A change of this type could also contribute to the alteration in the relationship of the conductance of the neomycin-induced state to transmembrane holding potential seen following the modification of RyR channel function by ryanodine.

The binding site for ryanodine is accessible only from the cytosolic face of the RyR channel (Tanna et al., 1998). Our observations would be consistent with a sequence of events in which the interaction of ryanodine with its high-affinity binding site on RyR produced conformational rearrangements throughout the conduction pathway of the channel,

resulting in a redistribution of charge at the luminal mouth of the channel and a decrease in the flexibility of the pathway. Although a steric interaction between bound ryanodine and neomycin within the conduction pathway of the channel may contribute to the prevention of neomycin permeation, such a mechanism is less likely to account for the reported alterations in the blocking characteristics of luminal neomycin.

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