Block of the Ryanodine Receptor Channel by Neomycin Is Relieved at High Holding Potentials

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ABSTRACT In this study we have investigated the actions of the aminoglycoside antibiotic neomycin on K^+ conductance in the purified sheep cardiac sarcoplasmic reticulum (SR) calcium-release channel (RyR). Neomycin induces a concentration- and voltage-dependent partial block from both the cytosolic and luminal faces of the channel. Blocking parameters for cytosolic and luminal block are markedly different. Neomycin has a greater affinity for the luminal site of interaction than the cytosolic site: zero-voltage dissociation constants ($K_b(0)$) are respectively 210.20 ± 22.80 and 589.70 ± 184.00 nM for luminal and cytosolic block. However, neomycin also exhibits voltage-dependent relief of block at holding potentials $>+60$ mV when applied to the cytosolic face and a similar phenomenon may occur with luminal neomycin at high negative holding potentials. These observations indicate that, under appropriate conditions, neomycin is capable of passing through the RyR channel.

INTRODUCTION

The interaction of endogenous intracellular polyamines such as spermine, spermidine, and putrescine with various surface membrane K^+ channels underlies the phenomenon of inward rectification (Oliver et al., 2000; Lopatin and Nichols, 2001). Investigations of the mechanisms involved in the interaction of this class of polycations with K^+ and other voltage- or ligand-regulated channels have revealed polyamines as concentration and voltage-dependent blockers of permeant ion translocation (Bähring et al., 1997; Haghighi and Cooper, 1998; Huang and Moczydlowski, 2001; Guo and Lu, 2000a, b). These studies have also established that endogenous polyamines are capable of blocking current when present at either the intracellular or extracellular surfaces of these channels and that the effectiveness of polyamines as blockers can be reduced at high transmembrane holding potentials as a result of translocation of the blocking ion through the channel.

Modification of ion channel function is also the underlying mechanism of toxicity of the polyamine aminoglycoside antibiotics neomycin, gentamicin, and streptomycin. Neomycin induces block in a variety of Ca^{2+} (Wagner et al., 1987; Suarez-Kurtz and Reuben, 1987; Duarte et al., 1993; Haws et al., 1996) and K^+ (Oosawa and Sokabe, 1986; Nomura et al., 1990) channels. A reduction in current flow through these channels by neomycin has been attributed to membrane charge effects (Suarez-Kurtz and Reuben, 1987), surface charge effects by competitive binding of the polycation to divalent cation binding sites (Haws et al., 1996), or pore occlusion (Winegar et al., 1996).

Neomycin is also known to inhibit the ryanodine receptor-mediated release of Ca^{2+} from isolated skeletal muscle sarcoplasmic reticulum (SR) vesicles (Palade, 1987; Calviello and Chiesi, 1989) and to disrupt communication between T-tubules and the SR in isolated skeletal muscle triad preparations (Yano et al., 1994). The mechanisms responsible for these actions are yet to be established.

In this communication we have investigated the possibility that the reported inhibition of Ca^{2+} release from the SR could result from block of the RyR channel by neomycin. We demonstrate that neomycin can induce a concentration- and voltage-dependent partial block of the RyR channel. Neomycin is capable of inducing these blocking events when applied to either the cytosolic or luminal face of the channel. Block appears to be dependent upon the molecule entering the pore of the open channel. In addition, our data indicate that block of the RyR channel by neomycin is relieved at high transmembrane holding potentials as the result of translocation of the polycation through the channel. The demonstration of permeability of a molecule considerably larger than the previously estimated minimum radius of the RyR conduction pathway raises questions about the dimensions of the conduction pathway and the selectivity filter of the RyR channel.

MATERIALS AND METHODS

Preparation of SR membrane vesicles

SR membrane vesicles were prepared as described previously (Sitsapesan and Williams, 1990). Sheep hearts were collected from a local farm and transported to the laboratory in ice-cold cardioplegic solution (Tomlins and Williams, 1986). All subsequent procedures were carried out at 4°C. The left ventricle and septum were homogenized in a solution containing 300 mM sucrose and 20 mM potassium piperazine-*N′N′*-bis-ethanesulfonic acid (PIPES) supplemented with a protease inhibitor cocktail (Sigma, Poole, UK). The homogenate was centrifuged at 7000 × *g* for 20 min and the pellet was discarded. The supernatant was then centrifuged for 45 min at 100,000 × *g*. The resulting pellet containing the mixed membranes was resuspended in a solution containing 400 mM KCl, 0.5 mM $MgCl_2$, 0.5

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mM $CaCl_2$, 0.5 mM 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 25 mM PIPES, pH 7.0, and 10% sucrose w/v, using a glass/PTFE tissue grinder. The same salt solution was used to make 20, 30, and 40% sucrose solutions for a discontinuous density gradient, onto which the mixed membrane suspension was layered before centrifugation at 100,000 × *g* for 120 min. Heavy SR (HSR) membrane vesicles were collected from the 30–40% interface and resuspended in 400 mM KCl and centrifuged for 45 min at 100,000 × *g*. The resulting pellet was resuspended in a solution containing 400 mM sucrose and 5 mM *N*′-2-hydroxyethylpiperazine-*N*′-2-sulfonic acid (HEPES) titrated to pH 7.4 using tris(hydroxymethyl)-methylamine (Tris), then snap-frozen in liquid nitrogen before storage at −80°C.

Solubilization and purification of the ryanodine receptor

The ryanodine receptor was purified as previously described (Lindsay and Williams, 1991), with some modifications, following solubilization with 3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS). HSR membrane vesicles were suspended in a solution containing 1 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA, and 25 mM Na-PIPES, pH 7.4, with NaOH plus 0.4–0.5% (w/v) CHAPS and 2–2.5 mg/ml L-α-phosphatidylcholine (PC), at a protein concentration of 2 mg/ml. This was incubated for 1 h on ice before sedimentation of the unsolubilized material at 100,000 × *g* for 45 min. Two samples of HSR membrane vesicles were prepared in this way, one containing 5 nM [^{3}H] ryanodine to enable detection of RyR at a later stage in the protocol.

RyRs were separated from the other solubilized protein components of the HSR by centrifugation on a 5–25% (w/v) linear sucrose gradient containing the same concentrations of CHAPS and PC used during the solubilization, with a 40% sucrose cushion. This was carried out overnight (16 h) at 100,000 × *g*. Two-milliliter gradient fractions were drawn from the base of the tubes, and the fraction containing RyR located by comparison with the sample incubated in the presence of [^{3}H] ryanodine. The purified RyR was then reconstituted into liposomes by a rapid dialysis technique against a total volume of 5 l of a buffer containing 0.1 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM EGTA, and 25 mM Na PIPES, pH 7.4, with NaOH. Dialysis was carried out by placing the sample in dialysis tubing into the outflow of a modified submersible pump, thus increasing the flow rate of buffer over the dialysis membrane. The sample was dialyzed for 4 h with two changes of buffer. The reconstituted receptor was diluted in an equal volume of 0.4 M sucrose before snap-freezing in liquid nitrogen and storage at −80°C.

Planar lipid bilayer methods

Planar phospholipid bilayers of phosphatidylethanolamine (in *n*-decane, 35 mg/ml) were painted across a 200-μm-diameter hole in a styrene copolymer partition separating two fluid-filled chambers referred to as *cis* (0.5 ml volume) and *trans* (1.0 ml volume). The *trans* chamber was held at ground, and the *cis* chamber was held at various holding potentials relative to ground using Ag-AgCl electrodes via 2% agar bridges in 3 M LiCl. Current flow was measured using an operational amplifier as a current-voltage converter (Miller, 1982). Bilayers were formed in solutions of 200 mM KCl and 20 mM HEPES, titrated to pH 7.4 with KOH to give a final K^+ concentration of 210 mM. Proteoliposomes were added to the *cis* chamber, and an osmotic gradient created by the addition of 100 μl of 3 M KCl to the *cis* chamber. Fusion of vesicles with the bilayer was achieved by further additions of KCl (50 μl) and by constantly stirring the solution in the *cis* chamber. After incorporation, the *cis* chamber was perfused with 210 mM K^+ to prevent further vesicle fusion. The RyR channel incorporates into the bilayer in a fixed orientation in such a way that the *cis* chamber corresponds to the cytosolic face of the channel, and the *trans* chamber corresponds to the luminal face (Sitsapesan and Williams, 1994a). Single channels were used in all experiments, as multiple channels could not be analyzed effectively. Channel open probability (P_o) was increased by adding 20–100 μM EMD41000, a caffeine analog (McGarry and Williams, 1994), to the *cis* chamber. Neomycin was then added to the solution in either the *cis* or *trans* chambers as required. The contaminating free-Ca^{2+} concentration of the solutions was monitored using a calcium-sensitive electrode as described previously (Sitsapesan and Williams, 1994b) and found to be ~10 μM. The experiments were performed at room temperature (21 ± 2°C).

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 200 Hz, and digitized at 4 kHz using an AT-based computer system (Intracel, Cambridge, UK). Neomycin induces the occurrence of a subconductance level distinct from the normal closed level. This means that subconductance events can be analyzed without contamination from normal channel closing events.

In this communication we have limited our analysis to a determination of the interactions of neomycin with open single RyR channels by monitoring transitions between the open state of the channel and the polycation-induced subconductance state. Such transitions are assumed to represent the entry and exit of neomycin to and from the open conduction pathway of the channel. Cursors for 50% threshold analysis were placed at the subconductance and fully open levels, and only sections of data where the channel was open (in the open or subconductance state) were analyzed (see Fig. 4 and text for details). This means that all data acquired using this method are for transitions between the normal channel open conductance level and the neomycin-induced subconductance level without interference from normal channel closings. These data have been used to monitor open probability (P_o) and dwell times in the open and subconductance states. By using these parameters we have analyzed the concentration and voltage dependence of the interaction of neomycin with open RyR channels.

Under the conditions used for analysis of open and subconductance state dwell times, the dead time was 1.4 ms. The impact of this was assessed in representative experiments. Following removal of events shorter than the dead time, individual dwell times of open and subconductance states were fitted to a probability density function using the method of maximum likelihood with a missed events correction, and the number of significant exponential components in the dwell time distributions were determined with the likelihood ratio test (Sitsapesan and Williams, 1994a). In all cases, distributions of dwell times were adequately described by single exponentials (differences in χ^2 between single and double components were not statistically significant). Time constants derived from these distributions were not significantly different from mean dwell times determined as the mean of all monitored events. Data are presented as mean ± SEM. Linear and nonlinear regression analysis was carried out using GraphPad Prism.

Materials

Neomycin is an aminoglycosidic antibiotic comprising a hexose ring surrounded by three amino sugars, as represented by the CPK models in Fig. 1. Neomycin has a net charge of +4.4 at pH 7.4 (Haws et al., 1996). The amino groups are distributed evenly across the molecule, with no concentration of positive charge at any particular location.

All solutions were prepared using de-ionized water produced by a Milli-Q water purification system. [^{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).

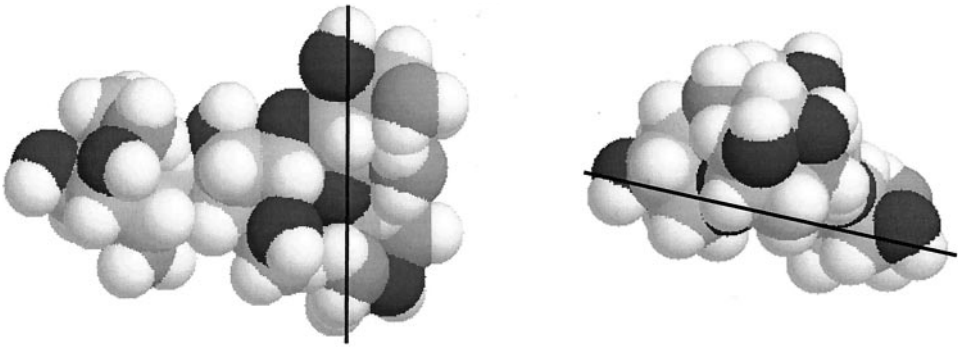

FIGURE 1 CPK models of the neomycin molecule. The two models represent different orientations of the polyamine, showing the maximum (*left*) and minimum (*right*) circular areas. In both cases the solid line is 10 Å. The models were created using molecular modeling software (Hyperchem, Hypercube Inc., Gainesville, FL), and were minimized using an MM+ force field.

RESULTS

Initial observations

In the absence of neomycin the RyR channel fluctuates between a nonconducting closed state and a single open state. In the presence of the polycation, fluctuations between the open and closed state of the channel are still apparent, together with additional fluctuations between the open state and a subconductance state. Examples of neomycin-induced subconductance states are shown in Fig. 2.

At an equivalent holding potential, the amplitude of the subconductance state induced by neomycin added at the cytosolic face of the channel differs from that induced by luminal addition of the polycation. In the representative traces shown in Fig. 2 at ±60 mV the amplitude of the subconductance state resulting from interaction of neomycin from the cytosolic face of the channel is ~4 pA, while the amplitude of the subconductance state induced by luminal neomycin is ~11 pA. The amplitude of the neomycin-induced subconductance states is not altered by changes in the concentration of the polycation (Fig. 2.).

Similarly, the amplitude of the neomycin-induced subconductance states is largely independent of holding potential. The relationship between subconductance state amplitude and holding potential is shown for several channels in Fig. 3. Neomycin is effective only from the side of the membrane to which it is applied. With neomycin present at the cytosolic face of the channel, subconductance states are seen only at positive holding potentials; with neomycin at the luminal face of the channel, subconductance states are only apparent at negative holding potentials. Following the addition of neomycin to the solution at the cytosolic face of the channel (Fig. 3, *A* and *B*), we observe no significant variation in subconductance state amplitude at holding po-

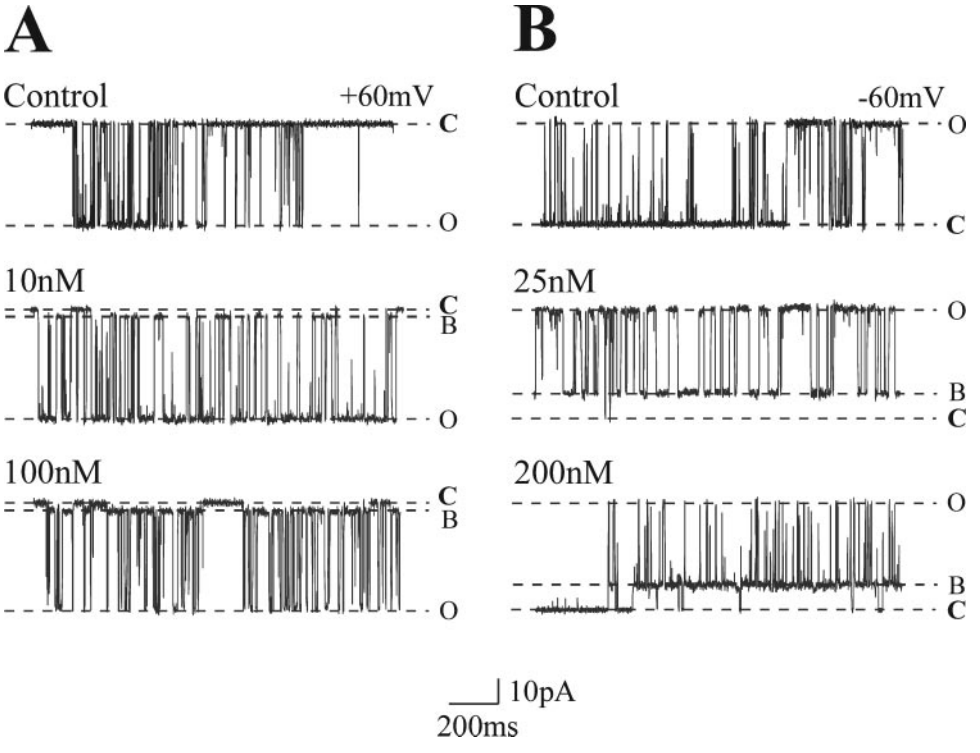

FIGURE 2 Representative single channel recordings showing a control purified RyR channel (*upper traces*), and the effect of the indicated concentrations of neomycin applied to either the cytosolic face (*A*) at +60 mV or the luminal face (*B*) at −60 mV. Neomycin induces a subconductance state in the open RyR channel and fluctuations can be seen between this subconductance state, indicating partial block, and the normal full open level. Neomycin-induced subconductance states within prolonged channel openings are interspersed with occasional normal opening events. It is notable that the amplitude of subconductance states induced by neomycin at the two channel faces differs. The amplitude of the subconductance states does not vary with changing neomycin concentration. The closed channel level is denoted by *C*, the normal open channel level by *O*, and the subconductance state that indicates neomycin block is labeled *B*.

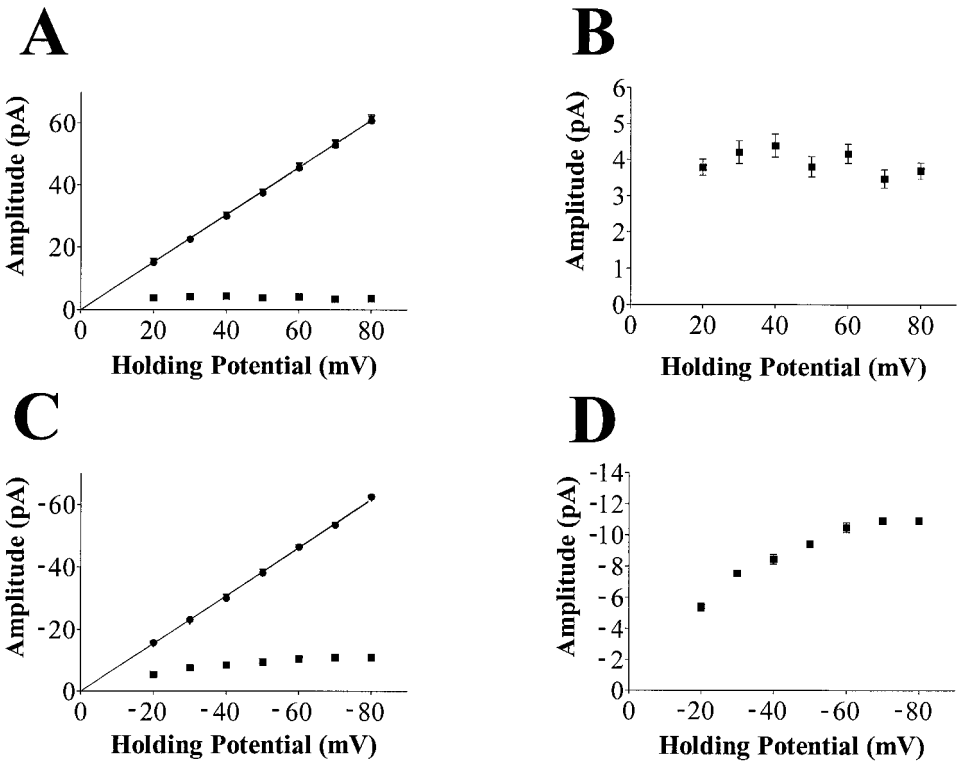

FIGURE 3 Current-voltage plots representing changes in the full open state and subconductance state amplitude with applied holding potential. (*A*) Full open state amplitudes in the absence of neomycin (●) together with subconductance state amplitudes (■) induced by the application of 100 nM to the solution at the cytosolic face of the channel. For clarity, variations in subconductance state amplitude with holding potential are shown on an expanded scale in (*B*). (*C*) Full open state amplitudes in the absence of neomycin (●) together with subconductance state amplitudes (■) induced by the application of 200 nM to the solution at the luminal face of the channel. Again, variations in subconductance state amplitude with holding potential are shown on an expanded scale in (*D*). The amplitude of the full open conductance state of the channel is unaltered by the presence of either cytosolic or luminal neomycin (▼) (*A* and *C*). In all cases $n \geq 4$.

tentials between +20 and +80 mV. Following the addition of neomycin to the solution at the luminal face of the channel we observe a small variation in subconductance state amplitude with increasing holding potential. Under these conditions subconductance amplitude increases from ~−5 pA to −11 pA as holding potential is increased from −20 to −80 mV (Fig. 3, *C* and *D*). The actions of neomycin are freely reversible when the site of application is perfused with the standard 210 mM K^+ recording solution (not shown).

Based on these initial observations we suggest that neomycin acts as a partial blocker of K^+ translocation in the open RyR channel. Neomycin has access to the open state of the RyR channel from either the cytosolic or luminal sides of the channel. Once bound, neomycin limits K^+ flux, resulting in the occurrence of characteristic subconductance states. Dwell times in the partially blocked states last several milliseconds.

We have investigated the factors that influence the interaction of neomycin with the open RyR channel using the sampling criteria described in Fig. 4. By limiting our analysis to transitions between the open and neomycin-induced states we are undoubtedly excluding other aspects of the modification of RyR channel function by this polycation; inspection of the traces in Fig. 2, *A* and *B* indicates that the channel can close during neomycin-induced subconductance events, and we cannot exclude the possibility that neomycin can interact with the closed channel. However, within the limits set out above, interactions of neomycin with RyR can be summarized as follows:

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

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 con-

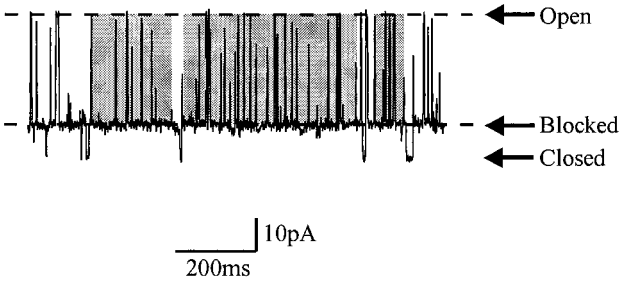

FIGURE 4 A representative single-channel recording to show the method of analysis used for the determination of the interaction of neomycin with the open RyR channel. Dotted lines represent the position of boundaries for 50% threshold analysis. The upper boundary is placed on the full open channel level "Open," and the lower boundary on the neomycin-induced subconductance "Blocked" level. The shaded regions show the portions of the trace in which transitions were analyzed. Closing events (either from the full open state or the neomycin-induced subconductance state "Closed") were not included in the analysis.

stants for the association of neomycin with and dissociation of neomycin from the 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.

The probability of occurrence of block is dependent upon neomycin concentration

We have carried out a rigorous examination of the influence of neomycin concentration on the probability of interaction between the polycation and the open RyR channel by monitoring the probability of occurrence of block (expressed as $1 - P_o$) for both cytosolic and luminal neomycin during prolonged periods of fluctuation between the open and blocked states, at holding potentials of +60 and −60 mV, respectively, as described in Materials and Methods. The relationships between $1 - P_o$ and neomycin concentration are displayed in Fig. 5. In both cases the relationship between the probability of occurrence of block within an opening and neomycin concentration can be described by a simple saturation curve of the form:

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

where $1 - P_o$ is the probability of occurrence of block, B_{max} is the maximum probability of occurrence of block, and K_m is the concentration of neomycin at which 50% of maximal occurrence of block is seen. The solid lines are best-fits to Eq. 1 obtained by nonlinear regression with the following parameters: cytosolic neomycin (Fig. 5 *A*) B_{max} 1.04 ± 0.03, K_m 41.74 ± 3.70 nM; luminal neomycin (Fig. 5 *B*) B_{max} 0.97 ± 0.05, K_m 25.89 ± 5.46 nM. These data indicate that the interaction of neomycin with the luminal site is of a higher affinity than that at the cytosolic site.

Consistent with the simplified scheme for the interaction of neomycin with the open RyR channel (Scheme 1) distributions of the dwell times in the open state and the neomycin-induced subconductance states are mono-exponential (see Materials and Methods for details). As a consequence, the apparent rate constants for the association of neomycin with the open RyR channel (k_{on}) and the dissociation of neomycin from the channel (k_{off}) can be determined as the reciprocal of the mean dwell time in the open state and subconductance state, respectively. In Fig. 6 we have plotted variations in k_{on} and k_{off} with neomycin concentrations ranging from 10 to 200 nM. Data for cytosolic block were obtained at +60 mV and data for luminal block at −60 mV. In both cases it is clear that in keeping with the proposed scheme, the major factor underlying the increased probability of occurrence of block with increasing concentrations of neomycin is a linear variation in the rate of association of the polycation with the channel. Linear regression gives slope values of 2.227 ± 0.087 s^{-1} nM^{-1} for cytosolic block and 2.506 ± 0.565 s^{-1} nM^{-1} for luminal block. By contrast, rates of dissociation of neomycin from either the cytosolic or luminal sites on RyR are influenced only weakly by polycation concentration. Linear regression gives slope values of 0.609 ± 0.088 s^{-1} nM^{-1} for cytosolic block and 0.158 ± 0.070 s^{-1} nM^{-1} for luminal block.

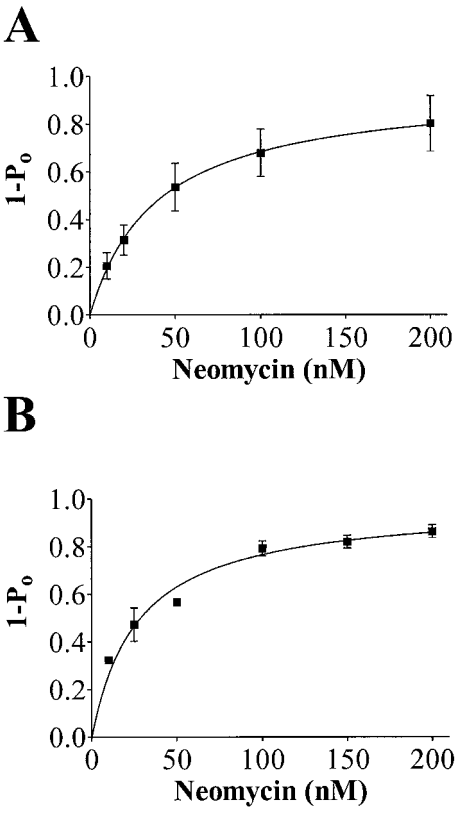

FIGURE 5 Influence of neomycin concentration on the probability of the occurrence of block ($1 - P_o$). Graph (*A*) represents increasing block induced by increasing neomycin concentration at the cytosolic face of the channel held at +60 mV. (*B*) Luminal block, measured at −60 mV. Lines are obtained from nonlinear regression, as described in the text. $n \geq 4$.

The probability of occurrence of neomycin block is dependent upon transmembrane holding potential

If the observed neomycin-induced subconductance states result from a partial block of K^+ translocation in RyR, a possible mechanism would involve the interaction of the polycation with sites within the conduction pathway of the channel and restriction of current flow. Such interactions are likely to take place within the voltage drop across the RyR

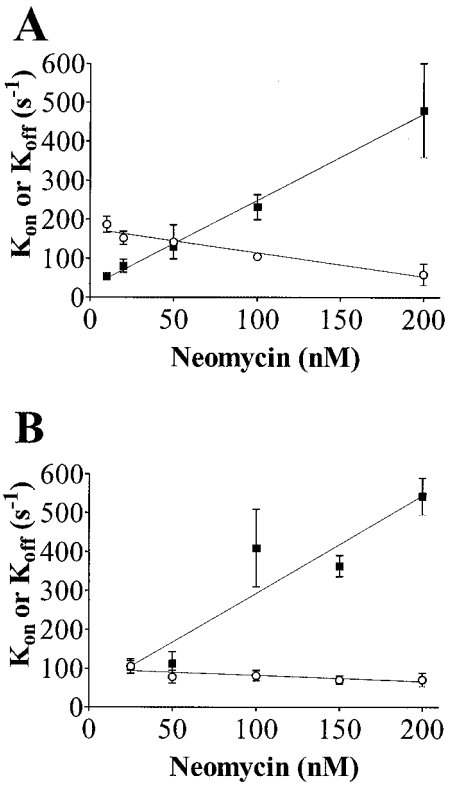

FIGURE 6 The relationship between increasing neomycin concentration and association (K_{on}, ■) and dissociation (K_{off}, ○) rates. (*A*) Cytosolic block, as measured at +60 mV; (*B*) luminal block, as measured at −60 mV. The increase in applied concentration results in changes in the rate of association for both the cytosolic and luminal interactions. Lines are derived from linear regression as described in the text. $n \geq 4$.

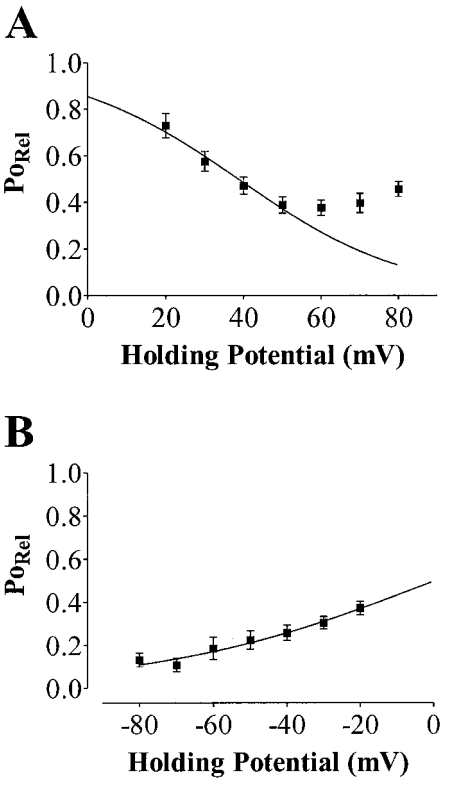

FIGURE 7 Graphs representing the influence of holding potential on P_o of the channel in the presence of 100 nM cytosolic (*A*) or 200 nM luminal (*B*) neomycin. Lines are derived from nonlinear regression calculated from the Woodhull model, as described in the text. In both cases the model has been used to predict a curve to show the deviation in the data from expected voltage dependence at higher holding potentials. $n \geq 4$.

channel and would be expected to be influenced by transmembrane holding potential.

We have investigated the putative voltage dependence of the interaction of neomycin from the cytosolic and luminal faces of the RyR channel within the context of a simple model first proposed by Woodhull (1973). With neomycin in the solutions at either the cytosolic or luminal face of the RyR channel, the probability of the channel being open in the presence of neomycin, defined as the relative open probability (P_{orel}), will be related to holding potential as follows:

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

where $z\delta$ is the effective valence of block, a product of the valence of neomycin (z) and the proportion of the voltage drop across the channel sensed by the polycation (δ), and $K_b(0)$ is the dissociation constant at a holding potential of 0 mV. *F*, *R*, and *T* have their usual meanings and *RT/F* is 25.2 mV at 20°C. We have monitored block of RyR by neomycin by adding the polycation to either the cytosolic side of the channel and determining P_{orel} at a range of positive holding potentials, or the luminal side of the channel and determining block at a range of negative holding potentials.

The relationship between P_{orel} and holding potential is shown with neomycin present at the cytosolic face of the channel in Fig. 7 *A* and with neomycin present at the luminal face of the channel in Fig. 7 *B*. The probability of occurrence of subconductance states within open events induced by both cytosolic and luminal neomycin is voltage-dependent. However, these relationships do not follow exactly those of other voltage-dependent blockers of RyR such as tetramethylammonium, tetraethylammonium, and tetrapropylammonium. These tetraalkylammonium cations are only effective as blockers of K^+ current in RyR when added to the cytosolic face of the channel. In addition, increasing transmembrane holding potential in the presence of cytosolic tetramethylammonium, tetraethylammonium, and tetrapropylammonium produces a progressive increase in the probability of block (Lindsay et al., 1991; Tinker et al., 1992b). This is clearly not the case when block is induced by neomycin present at the cytosolic face of RyR. Reminiscent of the situation with the tetraalkylammonium block-

ers we see a progressive decrease in P_{orel} as holding potential is raised from +20 to +50 mV, indicating that the probability of occurrence of blocking events within a channel opening increases as holding potential is made more positive. However, at holding potentials >+50 mV the probability of occurrence of blocking events appears to decline as P_{orel} increases between +50 and +80 mV (Fig. 7 *A*). The solid line in Fig. 7 *A* is the line of best fit for Eq. 2 obtained by nonlinear regression for the data obtained at +20, +30, +40, and +50 mV. The blocking parameters derived from this fit are as follows: $z\delta$ 1.05 ± 0.13 and $K_b(0)$ 589.70 ± 184.00 nM.

The probability of occurrence of block by luminal neomycin is also dependent upon transmembrane holding potential (Fig. 7 *B*). In this case P_{orel} shows a steady decline as holding potential is raised from −20 mV to −70 mV, indicating that the probability of occurrence of blocking events increases as holding potential is made more negative. It would appear that there is a slight increase in P_{orel} at −80 mV that may be analogous to, although not as marked as, the situation observed with cytosolic neomycin at high positive holding potentials (Fig. 7 *A*). Blocking parameters derived from the line of best fit for Eq. 2 obtained by nonlinear regression for the data obtained at −20 to −80 mV are $z\delta$ −0.66 ± 0.06 and $K_b(0)$ 210.20 ± 22.80 nM.

A comparison of the blocking parameters obtained from the Woodhull analysis of cytosolic and luminal neomycin block of RyR indicates that, in agreement with the information obtained by monitoring variations in block with changing neomycin concentration, the affinity of the luminal neomycin site of interaction on RyR is greater than that of the cytosolic site.

At least in the range of holding potentials from ±20 to ±50 mV, the influence of transmembrane holding potential is more marked for neomycin interacting with RyR from the cytosolic face of the channel. Although measurements of the effective valence of small monovalent tetraalkylammonium blocking cations have been used to define sites within the voltage drop of both RyR and other ion channels (French and Shoukimas, 1985; Moczydlowski, 1986; Tinker et al., 1992b), it is clear that it is not possible to define a specific value for δ in the values of $z\delta$ determined for cytosolic and luminal neomycin. We have no information on the relative location of the various cationic groups within the voltage drop or, for that matter, whether all these groups are present within the voltage drop at the range of holding potentials used in the determination of effective valence.

Information on the mechanisms underlying the modulation of neomycin block of RyR by holding potential can be obtained from an examination of variations in rates of association (K_{on}: reciprocal of mean dwell times in the open state) and dissociation (K_{off}: reciprocal of mean dwell times in the neomycin-induced subconductance state) with holding potential. If the rates of association and dissociation can

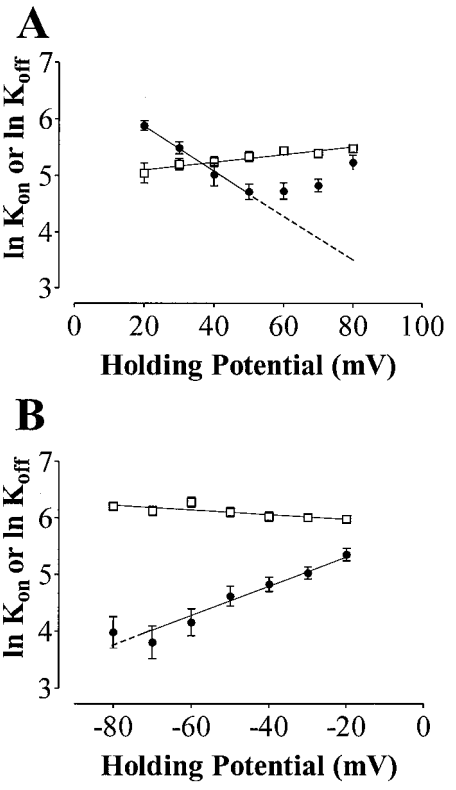

FIGURE 8 The relationship between holding potential and association (K_{on}, □) and dissociation (K_{off}, ●) rates for 100 nM cytosolic (*A*) or 200 nM luminal (*B*) block. It is apparent that association rate is affected weakly by changing holding potential, so the voltage dependence seen is reliant upon changing dissociation rate. All calculations are described in the text. Lines are derived from linear regression. $n \geq 4$.

be described by the Boltzmann relationship, then the rate constants at a given voltage (*V*) will be:

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

and

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

In these relationships $K(V)$ and $K(0)$ refer to the rate constants at a particular voltage and 0 mV, respectively, and *z* is the valence of the respective reaction. *R*, *T*, and *F* have their usual meanings; *z* and $K(0)$ can be determined from the slope and intercept of plots of the natural logarithm of K_{on} or K_{off} against holding potential. The overall voltage dependence of the reaction (z_{total}) is the sum of z_{on} and z_{off}. Plots of this form for cytosolic and luminal neomycin block of K^+ current in RyR are shown in Fig. 8.

The data displayed in Fig. 8 confirm and extend the observations presented in Fig. 7. An inspection of the variation in K_{on} and K_{off} for cytosolic neomycin (Fig. 8 *A*) demonstrates that the changes in the probability of occurrence of block with holding potential seen in Fig. 7 *A* result from changes in both the rate of association and the rate of

dissociation of the polycation. The influence of voltage on K_{on} is weak, with a small increase as holding potential is raised from +20 to +80 mV. However, transmembrane holding potential has a much more significant influence on K_{off}. An increase in holding potential from +20 to +50 mV is accompanied by a decrease in the rate of dissociation of neomycin from RyR. In contrast, the rate of dissociation of the polycation from RyR increases as holding potential is raised from +60 to +80 mV. These findings demonstrate that the deviation in the relationship between probability of occurrence of block and holding potential from the simple Woodhull model observed with neomycin at the cytosolic face of the RyR (Fig. 7 *A*) results largely from influences of voltage on the rate of dissociation of the blocker from the channel. The values of z_{on} and z_{off}, calculated from linear regression fits within the voltage range +20 to +80 mV for z_{on} and +20 to +50 mV for z_{off}, are 0.17 and 1.00, respectively, giving a z_{total} of 1.17, which is in good agreement with the total voltage dependence of the reaction determined from the Woodhull relationship at holding potentials between +20 and +50 mV in Fig. 7 *A*.

Plots of variation in ln K_{on} and ln K_{off} of luminal neomycin against holding potential are shown in Fig. 8 *B*. As is the case with cytosolic neomycin (Fig. 8 *A*), the most significant influence of holding potential on the block of RyR by luminal neomycin is on the rate of dissociation of the polycation from the channel; ln K_{off} decreases linearly as holding potential is increased from −20 to −70 mV. Consistent with the influence of holding potential on the probability of occurrence of block by luminal neomycin (Fig. 7 *B*), the rate of dissociation appears to show a slight increase at −80 mV. The value of z_{off} determined from the line of best fit obtained by linear regression over the entire range of holding potentials is −0.65. The rate of association of neomycin with RyR increases only very slightly as holding potential is raised from −20 to −80 mV. The value of z_{on} determined from the line of best fit obtained by linear regression over the entire range of holding potentials is −0.11. Thus, K_{off} is approximately six times more voltage-dependent than K_{on}, and the value of z_{total} determined from these plots is −0.76, compared to a value of −0.66 derived from the Woodhull relationship in Fig. 7 *B*.

DISCUSSION

The data presented in this communication demonstrate that neomycin can induce subconductance states in the open RyR channel. Unlike the vast majority of RyR blocking cations (Williams et al., 2001) neomycin is effective when present at either the cytosolic or luminal faces of the channel; however, the amplitudes of the subconductance states induced by cytosolic and luminal neomycin at equivalent holding potentials differ. The amplitude of the neomycin-induced subconductance state is independent of neomycin concentration and shows only slight variation with holding potential. The probability of occurrence of subconductance states induced by either cytosolic or luminal neomycin is influenced by both polycation concentration and holding potential. At high transmembrane holding potentials the probability of occurrence of subconductance states decreases as the result of an anomalous increase in the rate of blocker dissociation from RyR. Our working hypothesis is that subconductance states result from the interaction of neomycin with distinct sites accessible from either the cytosolic or luminal faces of the open RyR channel.

Mechanisms involved in the interaction of neomycin with RyR

In an earlier study we demonstrated that electrostatic forces play a major role in determining the interaction of a polycationic peptide derived from the NH_2-terminal domain of the *Shaker* K^+ channel with the RyR channel (Mead et al., 1998). This peptide is a concentration- and voltage-dependent blocker when added to the cytosolic face of the RyR channel. Increasing the net charge of the peptide from +3 to +7 resulted in an approximate 1000-fold increase in the affinity of the peptide. The alteration in affinity was mediated by a large increase in the rate of association of the peptide with the channel, indicating that the cytosolic mouth of the RyR channel carries negative charges located close to the peptide binding site that facilitate the interaction of the basic peptide with the conduction pathway to produce block. It is highly likely that this local negative electrostatic potential will influence the rate of association of a polycation such as neomycin with its cytosolic blocking site in RyR. A similar mechanism could be involved in the interaction of luminal neomycin with the RyR channel. Tu et al. (1994) demonstrated that application of carbodiimide to the luminal face of individual reconstituted cardiac RyR channels produced a significant reduction in unitary Cs^+ conductance. These authors proposed that the observed alteration in conductance resulted from a reduction in negative surface charge near the luminal mouth of the channel following the chemical neutralization of carboxyl groups.

How might the interaction of neomycin with RyR induce a subconductance state?

Open events with conductance less than the full open state are not uncommon events in voltage-clamped single RyR channels. Such events fall into two major categories, based on the manner in which the relative conductance of the event (conductance of event as a proportion of the full conductance event) is influenced by holding potential.

Subconductance states, in which relative conductance is independent of holding potential, have been reported to occur spontaneously in RyR (Liu et al., 1989) and following

the removal of FK506 binding proteins (Brillantes et al., 1994; Ahern et al., 1997; Kaftan et al., 1996; Xiao et al., 1997). Subconductance states also occur in response to the interaction of ligands with RyR. These include ryanodine and its derivatives (Rousseau et al., 1987; Tinker et al., 1996; Tanna et al., 1998), large tetraalkylammonium cations (Tinker et al., 1992c), and some local anesthetics (Tinker and Williams, 1993a). In all cases subconductance events are interspersed with openings to the full conductance state. A number of mechanisms have been proposed to account for the occurrence of subconductance states belonging to this category in both RyR and other ion channels. The ryanodine-induced subconductance state of RyR results from conformational alterations in the channel protein that modify the relative permeability of ions within the conduction pathway and the affinity of the channel for permeant ions (Lindsay et al., 1994). Ligand-induced conformational changes in channel structure have also been suggested as mechanisms responsible for the generation of subconductance states by H^+ in L-type Ca^{2+} channels (Pietrobon et al., 1989; Prod'hom et al., 1989) and by Zn^{2+} in cardiac Na^+ channels (Schild et al., 1991). Subconductance states in RyR induced by QX314, tetrabutylammonium, and tetrapentylammonium have been interpreted in terms of a partial occlusion of the conduction pathway arising from the introduction of an electrostatic barrier to ion translocation (Tinker and Williams, 1993a; Tinker et al., 1992c).

The second group of reduced conductance states arises as the result of occupation of the conduction pathway of the RyR channel by an impermeant or weakly permeant cation. As dwell times of individual blocking events are too short to be resolved, a time-averaged reduction of single-channel current is observed in the presence of these cations. With blocking cations in this group such as small tetraalkylammoniums (Lindsay et al., 1991; Tinker et al., 1992b) and procaine (Tinker and Williams, 1993a) the relative conductance of the blocked state is reduced as holding potential is increased. When reduced conductance results from the interaction of impermeant, voltage-dependent, blocking cations all opening events are to the reduced conductance state.

The reduced RyR conductance states induced by neomycin when it is added to either the cytosolic or luminal faces of the channel are unusual in that they display properties characteristic of both categories described above. In the presence of neomycin, openings to both the full conductance and a subconductance state are apparent; however, the relative conductance of the subconductance state decreases with increasing holding potential. Neomycin interacts with sites at both the cytosolic and luminal faces of the open RyR channel to induce a subconductance state; however, the influence of neomycin, once bound, on the translocation of ions through the channel varies with holding potential.

What other information do we have that might contribute toward an explanation of this phenomenon? An inspection of variations in rates of neomycin association with and dissociation from RyR indicate that, for both cytosolic and luminal sites, both rates are dependent upon the concentration of the polycation. In keeping with previous demonstrations of cation-induced subconductance states in voltage-dependent Ca^{2+} and Na^+ channels (Pietrobon et al., 1989; Prod'hom et al., 1989; Schild et al., 1991) such behavior is likely to indicate the involvement of a conformational change in the channel protein as a contributing factor in the alteration of channel conductance. The observed decrease in the relative conductance of subconductance states induced by both cytosolic and luminal neomycin with increasing holding potential indicates that an additional mechanism is involved in the total reaction. If, as is suggested above, the interaction of neomycin with either face of RyR results in a conformational change in the channel protein, the magnitude of this change may vary with holding potential. Similarly, the effectiveness of any putative neomycin-induced electrostatic barrier may vary with holding potential.

Is there evidence for partial occlusion of the conduction pathway by neomycin? An indication, but certainly not proof, of the likelihood of entry of a blocking cation into the conduction pathway of a channel can be obtained from an inspection of the influence of transmembrane holding potential on the probability of occurrence of the blocked state. With both cytosolic and luminal neomycin, this parameter is sensitive to variations in holding potential and is likely to reflect the movement of the charged ligand into and out of the voltage drop within the conduction pathway of the RyR channel. Movement of a large polycation such as neomycin into the voltage drop across the RyR channel would inevitably induce at least partial occlusion of the channel.

Our determinations of rates of association and dissociation of both cytosolic and luminal neomycin at varying holding potentials demonstrate that, while in both cases both rates are influenced by transmembrane potential, the bulk of the voltage dependence resides in the dissociation of the polycation from the channel. If, as we suggest, the voltage dependence of the association and dissociation processes reflects the translocation of the charged ligand to and from a site within of the voltage drop across the channel, then the observed asymmetry is likely to reflect differences in the physical location of the energy barrier governing association and dissociation of neomycin relative to the binding site of the polycation and the limit of the voltage drop at the entrance of the conduction pathway (Moczydlowski, 1986). The observed weak dependence of rates of association of both cytosolic and luminal neomycin on transmembrane potential suggests that these energy barriers are respectively near the cytosolic and luminal limits of the voltage drop across the channel.

Deviations from simple blocking schemes at high holding potentials

The relationships between the probability of occurrence of the neomycin-induced subconductance state (P_{orel}) and holding potential deviate from the predictions of the Woodhull scheme. Deviation is very marked with neomycin acting from the cytosolic face of the channel at high positive holding potentials and may be present at high negative holding potentials with neomycin at the luminal face of the channel. An inspection of rates of neomycin association and dissociation under these conditions reveals that this anomalous behavior results from increases in the rate of dissociation of the polycation at high holding potentials. Such behavior is characteristic of relief of block due to permeation of a blocking cation at high holding potentials (Tinker and Williams, 1995; Guo and Lu, 2000a, b; Huang and Moczydlowski, 2001).

Voltage-driven permeation of blocking polycations has been reported to occur in a number of cation-selective channels. Permeation-mediated relief of block has been observed with polyamines in glutamate receptor channels (Bähring et al., 1997), acetylcholine receptors (Haghighi and Cooper, 1998), Na^+ channels (Huang and Moczydlowski, 2001), cGMP-gated channels (Guo and Lu, 2000a), and inward-rectifier K^+ channels (Guo and Lu, 2000b). At high positive holding potentials, RyR is permeable to blocking monovalent and divalent derivatives of trimethylammonium; however, the magnitude of the observed relief of block is dependent upon valence. The divalent derivative is considerably more permeant than its monovalent equivalent (Tinker and Williams, 1995). This observation is likely to reflect the inherent higher relative permeability of divalent, as opposed to monovalent, cations in RyR. In earlier investigations we have suggested that conduction pathway of the RyR channel might contain a high density of negative charge that would facilitate the passage of divalent cations between binding sites (Tinker et al., 1992a). This putative arrangement of negative charge may also render the RyR conduction pathway permeable to polycations such as neomycin.

The minimum radius of the conduction pathway of the RyR channel

Previous studies have indicated that the minimum radius of the RyR channel conduction pathway is ~3.5 Å, and is located within the first 20% of the voltage drop across the channel that would be experienced by an ion entering from the luminal face of the channel (Tinker and Williams, 1993b). These investigations involved the determination of the relationship between the relative permeabilities of a wide range of organic monovalent cations and the minimum circular area of energy-minimized CPK equivalent models of the cations. Using an identical approach we have determined the minimum circular area of neomycin; this structure is shown together with the maximum circular area of the polycation in Fig. 1. The radius of the minimum circular area of neomycin is 5 Å. Based on our earlier determinations, neomycin is too large to go through the RyR channel. Voltage-driven permeation of RyR by neomycin suggests that, under appropriate conditions, the narrowest region of the RyR conduction pathway can display a degree of flexibility and allow the translocation of this large polycation through the channel. This possibility is considered further in the following communication, in which we examine the interactions of neomycin with ryanodine-modified RyR channels (Mead and Williams, 2002).

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