Interaction between Ryanodine and Neomycin Binding Sites on $Ca^{2+}$ Release Channel from Skeletal Muscle Sarcoplasmic Reticulum

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Neomycin is a potent inhibitor of skeletal muscle sarcoplasmic reticulum (SR) calcium release. To elucidate the mechanism of inhibition, the effects of neomycin on the binding of [$^3$H]ryanodine to the $Ca^{2+}$ release channel and on its channel activity when reconstituted into planar lipid bilayers were examined. Equilibrium binding of [$^3$H]ryanodine was partially inhibited by neomycin. Inhibition was incomplete at high neomycin concentrations, indicating noncompetitive inhibition rather than direct competitive inhibition. Neomycin and [$^3$H]ryanodine can bind to the channel simultaneously and, if [$^3$H]ryanodine is bound first, the addition of neomycin will slow the dissociation of [$^3$H]ryanodine from the high affinity site. Neomycin also slows the association of [$^3$H]ryanodine with the high affinity binding site. The neomycin binding site, therefore, appears to be distinct from the ryanodine binding site. Dissociation of [$^3$H]ryanodine from trypsin-treated membranes or from a solubilized 14 S complex is also slowed by neomycin. This complex is composed of polypeptides derived from the carboxyl terminus of the $Ca^{2+}$ release channel after Arg-4475 (Callaway, C., Seryshev, A., Wang, J. P., Slavik, K., Needelman, D. H., Cantu, C., Wu, Y., J ayaraman, T., Marks, A. R., and Hamilton, S. L. (1994) J. Biol. Chem. 269, 15876–15884). The proteolytic 14 S complex isolated with ryanodine bound produces a channel upon reconstitution into planar lipid bilayers, and its activity is inhibited by neomycin. Our data are consistent with a model in which the ryanodine binding sites, the neomycin binding sites, and the channel-forming portion of the $Ca^{2+}$ release channel are located between Arg-4475 and the carboxyl terminus.

The $Ca^{2+}$ release channel in the terminal cisternae of skeletal muscle allows the movement of $Ca^{2+}$ from the lumen of the sarcoplasmic reticulum (SR) to the cytoplasm in response to a signal from the transverse tubule (2). The protein that forms this channel can be activated by the binding of the plant alkaloid ryanodine at a high affinity site (3–5). The apparent affinity of [$^3$H]ryanodine for binding to the $Ca^{2+}$ release channel is dependent upon the functional state of the channel, and changes in binding of this ligand can be used to analyze and monitor the effects of modulators of $Ca^{2+}$ release channel function (6, 7). High affinity ryanodine binding sites are located in the protein between Arg-4475 and the carboxyl terminus (1).

Neomycin, a polycationic, aminoglycoside antibiotic (8), inhibits $Ca^{2+}$ release and blocks [$^3$H]ryanodine binding to the SR membranes (9–13). Wyskovsky et al. (13) reported that neomycin only blocks the fast component of the release while ruthenium red completely blocks the $Ca^{2+}$ efflux from SR vesicles, suggesting that inhibition by neomycin has a mechanism different from ruthenium red. The exact mechanism by which neomycin inhibits channel activity is unclear. Based on the effects of neomycin on the binding of [$^3$H]ryanodine to SR membranes and on $Ca^{2+}$ fluxes, Mack et al. (10) concluded that neomycin is competitive with ryanodine for high affinity binding sites. Furthermore, they suggested that neomycin at high concentrations slows the dissociation of [$^3$H]ryanodine from the high affinity site by an allosteric mechanism. In the present work, we examine the effects of neomycin on [$^3$H]ryanodine binding and on the behavior of the $Ca^{2+}$ release channel incorporated into planar lipid bilayers to demonstrate that ryanodine and neomycin bind noncompetitively to the channel.

EXPERIMENTAL PROCEDURES

Materials—[$^3$H]Ryanodine (61.5 Ci/mmol) was purchased from DuPont NEN. Ryanodine was obtained from Calbiochem. Neomycin sulfate was obtained from Sigma. Phosphatidylethanolamine (bovine heart) and phosphatidylserine (bovine brain) were obtained from Avanti Polar Lipids, Inc. Sarcoplasmic Reticulum (SR) Membrane Preparation—SR membranes were prepared from rabbit back and hindleg skeletal muscle and were purified by using sucrose gradient centrifugation as described elsewhere (14, 15). Protein was estimated by the method of Lowry et al. (16), using BSA as standard.

Trypsin Digestion SR Membranes and Purification of Solubilized [$^3$H]Ryanodine-binding Protein—SR membranes (3–6 mg) were incubated in 0.5 ml of Buffer I (0.3 M KCl, 100 $\mu$M $Ca^{2+}$, 20 mM MOPS, pH 7.4) with [$^3$H]ryanodine for 2 h at 37 °C. The concentration of [$^3$H]ryanodine is indicated in figure legends. SR membranes were then proteolyzed for 1 h at 37 °C by trypsin with a trypsin/protein ratio of 1:500. Proteolysis was stopped by soybean trypsin inhibitor at a trypsin/soybean trypsin inhibitor ratio of 1:10. Trypsinized SR membranes were pelleted by centrifugation in a Beckman Airfuge for 5 min at 30 p.s.i., then solubilized by resuspension in 2% digitonin, 0.3 M KCl, 100 $\mu$M $Ca^{2+}$, 20 mM MOPS, pH 7.4, followed by incubation for 30 min at 4 °C. The solubilized membrane proteins were partially purified by sedimentation through a 5–20% linear sucrose gradient in Beckman SW28 rotor for 18 h at 24,000 rpm. Fractions (20 drops) were collected from the bottom of the sucrose gradient and assayed for bound [$^3$H]ryanodine as described (1, 18).

Purification of the 14 S Complex of Proteolyzed SR $Ca^{2+}$ Release Channel—50 mg of sarcoplasmic reticulum membranes were pelleted at...
186,000 × g for 30 min to remove residual protease inhibitors and then the pellets were resuspended in 5 ml of 150 mM NaCl, 100 μM CaCl₂, 50 mM MOPS, pH 7.4. To trace label the receptor to follow its purification, [³H]ryanodine was added to a final concentration of 12 nM, and the membranes were incubated for 1 h at 37 °C. Unlabeled ryanodine was then added to 1 μM. 100 μM of trypsin was added, and the membranes were incubated for 1 h at 37 °C. The proteolysis was stopped by the addition of 1 mg of soybean trypsin inhibitor. Membranes were solubilized in 2% CHAPS for 30 min at 4 °C and centrifuged at 83,000 × g for 30 min to remove insoluble material. The supernatant was layered onto a 34-mi 5–20% sucrose gradient containing 0.4% CHAPS, 0.3M NaCl, 50 mM MOPS, pH 7.4, and centrifuged at 110,000 × g for 18 h. 50 drop fractions were collected, and the fractions containing the peak [³H]ryanodine counts were pooled, diluted to 50 mM NaCl, and applied to a 25-ml DEAE-trisacryl column and eluted with 100 mM NaCl, 0.4% CHAPS, 20 mM MOPS, pH 7.4. Following a second sucrose gradient, the peak fractions were pooled and used for binding and bilayer studies. The channel activity of this 14 S [³H]ryanodine-labeled Ca²⁺ release channel proteolytic fragment was determined by the bilayer techniques described below.

Equilibrium [³H]ryanodine Binding—[³H]ryanodine was incubated overnight (15–17 h) at room temperature (23 °C) with 5–75 μg of SR membranes in 100–250 μl of buffer containing 0.3 mM KCl, 20 mM MOPS, pH 7.20, 100 μM free Ca²⁺, 100 μg/ml bovine serum albumin (BSA), 200 μM phenylmethysulfonyl fluoride, 200 μM aminobenzamidine, 1 μM each of aprotinin, leupeptin, and pepstatin A. Non-specific binding was defined by the presence of either 10 μM or 100 μM ryanodine as indicated in the figure legends. The incubation was ended by rapid filtration of the entire sample volume through Whatman GF/F glass fiber filters and washed with 5 × 5 ml of ice-cold wash buffer containing 0.3 mM KCl, 100 μM Ca²⁺, and 10 mM MOPS, pH 7.2. The radioactivity bound to the filters was quantitated by liquid scintillation counting.

[³H]ryanodine Association Kinetics—The association experiments were initiated by adding SR membranes (0.1 mg of protein with 31 pmol/mg binding sites) to 10 ml of buffer containing 0.3 mM KCl, 20 mM MOPS, pH 7.4, 100 μM free Ca²⁺, and 1 mM AMP-PCP, 1 μM BSA, and protease inhibitors in the presence and absence of 100 mM neomycin. At the indicated times, 0.1–0.1 μl aliquots were filtered, washed, and processed for liquid scintillation counting as described above.

[³H]ryanodine Dissociation Kinetics—SR membranes (0.4–0.5 mg of protein with 5–6 pmol/mg binding sites) were equilibrated with 40 nm [³H]ryanodine in 0.3 mM KCl, 20 mM MOPS, pH 7.4, 100 μM free Ca²⁺, 100 μM bovine serum albumin (BSA), and protease inhibitors at temperature and times indicated in figure legends. The dissociation of [³H]ryanodine was initiated by diluting the membranes more than 400 fold in the same buffer and was carried out at room temperature. Aliquots were filtered at the time indicated in the figure. The amount of radioactivity bound to the filters was quantitated by scintillation counting.

Dissociation of [³H]ryanodine from proteolyzed SR membranes was performed as follows: SR membranes were incubated with 40 nm [³H]ryanodine in Buffer I (0.3 mM KCl, 100 μM Ca²⁺, and 20 mM MOPS, pH 7.4) at 37 °C for 2 h, then proteolyzed by trypsin (1:500 trypsin:SR membranes) at 1 h at 37 °C. The membranes were solubilized in 2% digitonin, and the 14 S complex was isolated using sucrose gradient centrifugation. Aliquots (440 μl) of the 14 S complex were diluted into 10 ml of Buffer I in the presence and absence of 20 μM neomycin. Dissociation was performed at room temperature, and aliquots (0.4 ml) of diluted sample were added to 200 μl of ice-cold Buffer I containing rabbit γ-globulin (5 mg/ml), BSA (5 mg/ml), and 10% polyethylene glycol (PEG) 8000. After 15 min of incubation at 4 °C, the samples were filtered and washed with 5 × 5 ml of ice-cold wash buffer containing 10% polyethylene glycol.

Bilayer Techniques—Planar bilayers consisting of 1:1 L-[³H]ryanodine and L-[³H]ryanodine labeled Ca²⁺ release channel were formed following the Mueller-Rudin procedure across a 100-μm diameter aperture in teflon cups as described previously (17). The mixture of phospholipids was dissolved in n-decane (Sigma) at a concentration of 25 mg/ml. Both chambers were filled with buffer solution (25 mM Cs₂SO₄, 10 mM MOPS, and 8 μM CaCl₂, pH 7.4). After bilayer formation, 5 μl of SR membranes were added to the cis chamber to give a final protein concentration of 0.5 mg/ml. The other side of the bilayer was defined as trans. An osmotic shock was performed between the cis and trans chambers by adding a concentrated salt solution (1 mM Cs₂SO₄) in the cis chamber. Recording solutions contained 225 mM Cs₂SO₄ in the cis chamber and 25 mM Cs₂SO₄ in the trans chamber. All subsequent additions were made to the cis chamber. Agar/KCl bridges were used to connect the chambers to AgAgCl electrodes immersed in 2 mM KCl. Holding potential was +40 mV. The data were filtered at 2.5 kHz and digitized at 10 kHz. Analysis was made using pCLAMP software: programs CLAMPEX, FETCHAN, and pSTAT (Axon Instruments, Inc). To facilitate incorporation and detection of channels from the 14 S complex, K⁺ was used as a current carrier, and all solutions contained KCl at the same concentrations used for Cs₂SO₄.

Data Analysis—[³H]ryanodine binding data were analyzed by nonlinear curve-fitting using Sigma Plot (Jandel Scientific). Non-specific binding was subtracted prior to analysis.

Association kinetics:

\[ B_i = \sum_{n=0}^{\infty} A_i^n \frac{(1 - \exp(-t \alpha_i))}{\alpha_i^n} \]

where \( B_i \) = bound ligand at time t, \( \alpha_i \) = time after addition of [³H]ryanodine, \( n \) = number of components, \( A_i \) = amount of ligand bound to component i = \( 1/K_{app} \) n = 3.

Dissociation kinetics:

\[ B_i = \sum_{n=0}^{\infty} A_i^n \exp(-t \alpha_i) \]

where \( B_i \) = bound ligand at time t, \( n \) = number of components, \( A_i \) = amount of bound ligand to component i at t = 0, \( \alpha_i = 1/K_{ass} \)

Model 1. Competitive interaction between neomycin and ryanodine at the high affinity binding site.

\[ K_{app} = K_d \cdot \frac{L}{K_d} \]

Model 2. Noncompetitive binding of neomycin allosterically alters the high affinity ryanodine binding site.

Inhibition of [³H]ryanodine equilibrium binding by neomycin was analyzed by nonlinear least squares fitting to the following equation:

\[ B_i = \frac{A}{1 + K_{app} + B_j} + B_j \]

where \( B_j \) is the observed binding in the presence of the inhibitor concentration, \( B_j \) is the bound [³H]ryanodine concentration at maximal inhibition, \( A \) is the inhibitable binding and is equal to \( B_j - B_j \) and \( B_j \) is the bound [³H]ryanodine at zero inhibitor concentration. In Model 1, for competitive binding, \( B_j \) equals nonspecific binding, and

\[ K_{app} = K_d \cdot \frac{L}{K_d} \]

and \( L = [\text{free} \ [³H]ryanodine] \)

From the cyclic model (2) of allosteric inhibition, \( B_j \) and \( B_j \) reflect the binding of [³H]ryanodine in the absence and presence of inhibitor, respectively.

Thus

\[ B_j = \frac{B_j}{1 + K_{ass} + B_j} \]

where \( L \) is the [³H]ryanodine concentration and \( K_{ass} \) is the equilibrium dissociation constant for [³H]ryanodine and \( B_j \) is the total binding site concentration. Likewise

\[ B_j = \frac{B_j}{1 + K_{ass} + B_j} \]

and

\[ K_{ass} = \frac{K_{ass} \cdot K_d}{L + K_{ass} \cdot K_d} \]

Thus, from the values of \( B_j \), \( B_j \), and \( K_{ass} \) (determined by curve-fitting),
and the known values for $K_{d1}$ and $L$, the binding constant for $[^3H]ryanodine$ in the presence of inhibitor, $K_{d2}$, can be derived. These equations will also yield the values for $K_1$ and $K_2$, the dissociation constants of the inhibitor in the absence and presence of $[^3H]ryanodine$, respectively.

**RESULTS**

Neomycin is a potent inhibitor of $[^3H]ryanodine$ binding and of Ca$^{2+}$ release channel activity (10–13). It has been proposed that neomycin blocks channel activation by ryanodine by competitively binding at the same site as ryanodine (10, 11). To investigate this mechanism more closely, the effects of neomycin on $[^3H]ryanodine$ binding were examined in detail. The $[^3H]ryanodine$ concentrations were kept low (1–20 nM) in these experiments to maintain binding exclusively at the high affinity ryanodine binding site. This avoids contributions from binding to low affinity sites. The inhibition of $[^3H]ryanodine$ binding by varying concentrations of neomycin was determined at four concentrations of $[^3H]ryanodine$ (Fig. 1A). At each concentration, inhibition was incomplete and reached a plateau ranging from 43% to 74% of the displaceable binding. The inhibition by neomycin is inconsistent with simple competitive inhibition at the $[^3H]ryanodine$ binding site. Competitive inhibition requires complete displacement of $[^3H]ryanodine$ to nonspecific binding sites. The inhibition of $[^3H]ryanodine$ binding by neomycin to 23 nM in 10 nM neomycin and to 46 nM in 10 μM neomycin.

While the data of Fig. 1, A and C, are inconsistent with simple competitive binding, the data can be analyzed in terms of a cyclic model for allosteric, noncompetitive inhibition (see “Experimental Procedures”). The data of Fig. 1A are well fit by Equation 3 as shown by the solid lines. From the values of $B_p$, $B_o$, and $K_{app}$ obtained by nonlinear least squares fitting and the affinity of $[^3H]ryanodine$ (14 nM) and using the equations described under “Experimental Procedures,” the binding affinity for neomycin was calculated to be 300 nM and 1100 nM in the absence and presence of ryanodine, respectively (Table 1). Likewise, the affinity of $[^3H]ryanodine$ in the presence of high concentrations of neomycin was calculated to be 50 nM, consistent with the value of 46 nM determined by direct binding isotherm in the presence of 10 μM neomycin in Fig. 1C.

To further explore the interaction between ryanodine binding and neomycin binding, the kinetics of association and dissociation in the presence of $[^3H]ryanodine$ were examined. If there is no effect on association rate, then the binding cannot be competitive. Changes in the rate of association can, however, occur with either a competitive or a noncompetitive mechanism. The association of $[^3H]ryanodine$ (Fig. 2) is characterized by a single component ($k_0 = 0.0025$ min$^{-1}$ M$^{-1}$). Neomycin decreases the apparent rate of association to 0.00041, a 6.1-fold effect. The predicted dissociation rate constant from the $k_{obs}$ plot is 0.011 min$^{-1}$ and does not appear to change significantly in the presence of neomycin.

Neomycin also slows the dissociation rate of bound $[^3H]ryanodine$ from the site (Fig. 3), a finding consistent only with a noncompetitive interaction between the neomycin and the ryanodine binding sites. In the absence of neomycin, the dissociation data were fit with 3 descending exponential components ($k_{-1} = 0.013$ min$^{-1}$, $k_{-2} = 0.0026$ min$^{-1}$, and $k_{-3} = 0.00045$ min$^{-1}$). The relative amounts of each of these components varied only slightly among membrane preparations. In 12 preparations, the fast component constituted 25.0 ± 4.2% (mean ± S.E.), the intermediate 70.1 ± 3.6%, and the slow component 5.7 ± 1.8% of the dissociation. There were no statistically significant changes in the relative amounts of these components with different initial occupancies by $[^3H]ryanodine$ (data not shown).

The data obtained from dissociation experiments in the presence of 10 μM neomycin were also fit with the same exponential rates in 8 separate experiments. The fast component was 2.6 ± 1.0%, the intermediate 25.7 ± 9.1%, and the slow component 71.8 ± 8.7% of the binding. Each value was significantly different from the corresponding control value. The presence of neomycin in the dissociation buffer decreases the relative amounts of the fast and intermediate components of the dissociation and increases the amount of slow component. The rate constant for the fast component is similar in magnitude to that predicted from the
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Calculation of $K_{i1}$ and $K_{i2}$ from the data in Fig. 1A

| Neomycin $K_d = 310 \text{ nm}$; $K_i = 1100 \text{ nm}$ in high ryanodine. |
|-----------------|---|---|---|---|---|
| Value | 1 | 2 | 3 | 4 | Units |
| $L$ | 1.68 | 5.0 | 10.0 | 20.0 | nm |
| $N_{i0}^a$ | 0.08 | 0.18 | 0.26 | 0.40 | pmol/mg |
| $B_0$ | 1.72 | 4.01 | 6.22 | 7.78 | pmol/mg |
| $B_0 - N_{i0}$ | 1.64 | 3.83 | 5.96 | 7.38 | pmol/mg |
| $B_1$ | 0.52 | 1.35 | 2.52 | 4.18 | pmol/mg |
| $B_1 - N_{i0}$ | 0.44 | 1.17 | 2.26 | 3.78 | pmol/mg |
| $K_{i0}$ | 282 | 385 | 456 | 525 | nm |
| $K_{i1}$ | 15.3 | 14.5 | 14.3 | 12.5 | pmol/mg |
| $K_{i0}/K_{i1}^c$ | 4.05 | 4.06 | 3.80 | 3.30 | |
| $K_{i2}^d$ | 1050 | 1250 | 1200 | 1022 | pmol/mg |
| $K_{i3}^d$ | 260 | 308 | 316 | 309 | pmol/mg |

$^a$ $N_{i0}$ = nonspecific binding.
$^b$ $B_0 = (B_0 - N_{i0}) (1 + K_{i0}/L)$.
$^c$ $K_{i0} = L / B_0 - N_{i0}$.
$^d$ $K_{i2} = K_{i0}/(L + K_{i1}/K_{i2}/K_{i3}/(L + K_{i1}))$.

**FIG. 2.** Effect of neomycin on the association of $[3H]$ryanodine to rabbit skeletal muscle SR membranes. SR membranes (0.1 mg) were added to 10 ml of 0.3 M KCl, 20 mM MOPS, pH 7.4, 100 mM Ca$^{2+}$, 2.5–20 nm $[3H]$ryanodine, 0.1 mg/ml BSA, 1 mM AMP-PCP, and protease inhibitors in the absence () and presence (○) of 100 nm neomycin. At various times at room temperature, 20-$\mu$L aliquots were filtered, and bound $[3H]$ryanodine was determined as described under “Experimental Procedures.” Dissociation was initiated by diluting more than 400-fold in binding buffer plus (●) or minus (○) 10 $\mu$m neomycin. Dissociation experiments were performed at room temperature. After subtraction of nonspecific binding, the data were fit to a three-component exponential decay (solid lines) with rate constants indicated in the text. B is the specific counts/min bound at time t.

**FIG. 3.** Effect of neomycin on the rate of dissociation of $[3H]$ryanodine from SR membranes. SR membranes (0.4–0.5 mg of protein) were equilibrated with 40 nm $[3H]$ryanodine under conditions described under “Experimental Procedures.” Dissociation was initiated by diluting more than 400-fold in binding buffer plus (●) or minus (○) 10 $\mu$m neomycin. Dissociation experiments were performed at room temperature. After subtraction of nonspecific binding, the data were fit to a three-component exponential decay (solid lines) with rate constants indicated in the text. B is the specific counts/min bound at time t.

These data again support a noncompetitive interaction between neomycin and ryanodine.

Previous work from this laboratory has shown that both high and low affinity ryanodine binding sites are located in a 14 S proteolytic complex composed only of polypeptides derived from the carboxyl terminus after Arg-4475 in the primary sequence of the Ca$^{2+}$ release channel (1). In SR membranes (not solubi-
dissociation of $[^3H]$ryanodine (bound prior to proteolysis) nor eradicate the 14 S complex does not significantly alter the rate of proteolysis with trypsin under the conditions which generated ryanodine receptor is characterized by two components containing bound $[^3H]$ryanodine. The sucrose gradient profile of the proteolyzed) solubilized and purified forms of the protein, each labeled from the sucrose gradient was diluted into binding buffer at 37 °C. The membranes were proteolyzed by trypsin (trypsin/protein ratio is 1:500) at 37 °C for 1 h. Proteolysis was stopped with a 10-fold excess soybean trypsin inhibitor. Both proteolyzed and nonproteolyzed SR membranes were solubilized in 2% digitonin, 0.3 mM KCl, 100 mM Ca$^{2+}$, 20 mM MOPS, pH 7.4, for 30 min at 4 °C, then sedimented on a 5–20% sucrose gradient as described under “Experimental Procedures.” ○, gradient profile of 30 S complex (nonproteolyzed); ●, gradient of 14 S complex (proteolyzed). Fractions range from the bottom to the top of the gradient.

To determine whether the neomycin binding site is on the 14 S complex, we generated the 30 S (intact) and the 14 S (proteolyzed) solubilized and purified forms of the protein, each containing bound $[^3H]$ryanodine. The sucrose gradient profile is shown in Fig. 6. We then determined the effect of neomycin on the rate of dissociation of $[^3H]$ryanodine from the purified 30 S and 14 S complexes (Fig. 7, A and B). Similar to the results obtained from SR membranes, 20 mM neomycin slows $[^3H]$ryanodine dissociation from both the 30 S intact Ca$^{2+}$ release channel (Fig. 7A) and 14 S complex (Fig. 7B). Dissociation of $[^3H]$ryanodine from the detergent-solubilized and gradient-purified ryanodine receptor is characterized by two components (k$_{-1}$ = 0.016 min$^{-1}$, k$_{-2}$ = 0.0063 min$^{-1}$), both of which are faster than those observed in intact membranes (Fig. 3). Dissociation from 14 S is even faster and is best fit by 3 components (k$_{-1}$ = 0.026 min$^{-1}$, k$_{-2}$ = 0.011 min$^{-1}$, k$_{-3}$ = 0.0004 min$^{-1}$).

The question of whether the 14 S complex still behaves as an ion channel was addressed by reconstitution of the 14 S complex into planar lipid bilayers. To obtain a higher purity for the 14 S complex, the fraction purified by a sucrose gradient and the DEAE-column was further purified on a second sucrose gradient as described under “Experimental Procedures.” ○, control; ●, 20 mM neomycin.

Fig. 5. Effect of neomycin on the activity of Ca$^{2+}$ release channel reconstituted into planar lipid bilayer. SR membranes were added to the cis chamber, as described under “Experimental Procedures.” The channel modulators were also added to the cis chamber. The traces are representative recordings of one of three independent experiments. Traces a and b, effect of neomycin on Ca$^{2+}$ release channel; a, control; b, 5 μM neomycin (P0 decreases in this experiment from 0.32 to 0.03). Traces c–e, effect of neomycin on ryanodine-modified Ca$^{2+}$ release channel; c, control; d, 2 μM ryanodine; e, 2 μM ryanodine, 10 μM neomycin. Upward deflections represent channel openings.

Fig. 6. Sedimentation of $[^3H]$ryanodine-labeled proteins solubilized from nontrypsinized and trypsinized SR membranes. SR membranes (6 mg) were incubated with 50 nM $[^3H]$ryanodine for 2 h in binding buffer at 37 °C. The membranes were proteolyzed by trypsin (trypsin/protein ratio is 1:500) at 37 °C for 1 h. Proteolysis was stopped with a 10-fold excess soybean trypsin inhibitor. Both proteolyzed and nonproteolyzed SR membranes were solubilized in 2% digitonin, 0.3 mM KCl, 100 mM Ca$^{2+}$, 20 mM MOPS, pH 7.4, for 30 min at 4 °C, then sedimented on a 5–20% sucrose gradient as described under “Experimental Procedures.” ○, gradient profile of 30 S complex (nonproteolyzed); ●, gradient of 14 S complex (proteolyzed). Fractions range from the bottom to the top of the gradient.

Fig. 7. A, dissociation of $[^3H]$ryanodine from isolated 30 S (unproteolyzed) complex. The purified $[^3H]$ryanodine-labeled 30 S complex isolated from the sucrose gradient was diluted into binding buffer at room temperature to initiate $[^3H]$ryanodine dissociation. Aliquots were filtered at the indicated times as described under “Experimental Procedures.” ○, control; ●, 20 mM neomycin. B, dissociation from the 14 S complex. The $[^3H]$ryanodine-labeled SR membranes were trypsinized, and the binding protein was purified on a 5–20% sucrose gradient as described under “Experimental Procedures.” The 14 S complex was diluted into Buffer I at room temperature to initiate dissociation. ○, control; ●, 20 mM neomycin.

Fig. 8. Silver-stained gel in Fig. 8B. A Western blot with an antibody to the last 9 amino acids is shown in Fig. 8C. The major components of the 14 S complex (labeled a–e) have previously been identified by Callaway et al. (1) as the 76 kDa (4476-carboxyl terminus) (a), 66 kDa (a cleavage product of the 76 kDa containing the carboxyl-terminal residues) (b), 56 kDa (calseque-
trin) (c), 37 kDa (derived from N terminus of 76 kDa and recognized by an antibody to residues 4670–4685) (d), and 27 kDa (beginning with 4756 and containing the carboxyl terminus) (e). In this preparation, the identity of the 76-kDa, the 56-kDa, and the 27-kDa peptides were confirmed by amino-terminal sequencing. All other bands were identified in Western blots as described previously. The 14 S complex isolated with [³H]ryanodine bound to the high affinity site was reconstituted into planar lipid bilayers, and the channel activity was monitored. In Fig. 9, 3 channels were incorporated, each with a conductance of 570 pS in 225 mM KCl (Fig. 9A). Neomycin caused the channel to close more frequently (Fig. 9B). Similar channels were seen in 6 different 14 S preparations and in a total of 8 trials.

**DISCUSSION**

The data presented here demonstrate that the channel-forming regions of the channel, the ryanodine binding sites, and the neomycin binding sites are all located between Arg-4475 and the carboxyl terminus of the Ca²⁺ release channel, but that the neomycin binding site is distinct from that of ryanodine. Neomycin inhibits [³H]ryanodine binding and the activity of both the intact channel and the ryanodine-modified channel in a manner consistent with neomycin binding being noncompetitive with ryanodine binding.

To demonstrate clearly the existence of a distinct binding site for neomycin, it was necessary to show that the binding was inconsistent with competitive binding to the ryanodine binding sites. Competitive inhibition can be distinguished from noncompetitive binding by analysis of equilibrium binding and by kinetic experiments. Since noncompetitive inhibition is mediated by allostERIC effects, the result is a decrease in binding affinity with concomitant changes in the association rate, dissociation rate, or both. The change in affinity is saturable with inhibitor concentration as the inhibitor site becomes fully occupied. Competitive binding should reveal a change in apparent affinity that is not saturable with increasing inhibitor concentration and have no changes in the dissociation rate. Neither competitive nor noncompetitive inhibition will display a change in the number of binding sites.
The data in Fig. 1 clearly show incomplete inhibition of \(^{3}H\)ryanodine binding by neomycin, which is inconsistent with competitive inhibition but fully consistent with noncompetitive inhibition. Therefore, the effect of neomycin is to change the affinity of ryanodine about 4-fold while ryanodine has a reciprocal 4-fold effect on the affinity of neomycin. The affinity of neomycin for the ryanodine receptor is about 300 nM. The kinetic data further support noncompetitive inhibition of \(^{3}H\)ryanodine binding by neomycin. At a high concentration of 100 \(\mu M\), neomycin decreases the association rate of \(^{3}H\)ryanodine dramatically. (If neomycin was competitive with ryanodine, the association rate should decrease proportionally with the predicted occupancy of neomycin. With a \(K_{d} = 300 \text{nm} \), the association rate should have decreased about 300-fold but actually changed only 6.1-fold.) More definitive is the analysis of the dissociation rates. A competitive inhibitor should not affect the dissociation rate under conditions where there is no rebinding of ligand. However, neomycin clearly inhibits dissociation of \(^{3}H\)ryanodine and alters the proportion of the three distinct rates of dissociation toward the slower components. Slowing of both association and dissociation rates is consistent with allosteric effects. Since the effect on the association rate constant is stronger, the net result is a decrease in ryanodine binding affinity at equilibrium.

Inhibition of \(^{3}H\)ryanodine binding by neomycin was interpreted by Mack et al. (10) as competitive. However, their data are not discrepant with the data presented here and are fully consistent with an allosteric model for noncompetitive inhibition. They also display data demonstrating slower dissociation of \(^{3}H\)ryanodine in the presence of high concentrations of neomycin, a result incompatible with a simple competitive mechanism.

Further support for a model wherein neomycin binds a site distinct from the ryanodine binding site is obtained from functional assays of channel activity in planar lipid bilayers. Ryanodine alone promotes long open states with a lower conductance than seen in the absence of ryanodine. The further addition of neomycin produces frequent fast closings: the mean open time is decreased. However, the channel opens to the conductance level seen in the presence of ryanodine, never to the higher conductance level characteristic of the unbound channel. If neomycin acted by competitive displacement of ryanodine, the channel would be expected to occasionally reopen to the unmodified level and this is not observed. Ryanodine, thus, appears to remain bound in the presence of neomycin, inhibition of channel activity. The effect of neomycin on the affinity of \(^{3}H\)ryanodine does not directly account for inhibition of the ryanodine-modified channel. The mechanism of neomycin inhibition may be through stabilization of a closed conformation or by direct channel block.

Neomycin slows the dissociation of \(^{3}H\)ryanodine from the purified Ca\(^{2+}\) release channel (30 S) and a 14 S complex which we have previously shown to be composed of peptides derived from the carboxyl terminus after Arg-4475 (1). The ryanodine-modified 14 S complex purified after trypsin digestion forms a channel in the bilayer, and this activity is inhibited by neomycin. This is consistent with the slowing of the dissociation of \(^{3}H\)ryanodine from its binding site on the 14 S complex by neomycin. It is extremely difficult to eliminate the possibility that a minor contaminant of this preparation is forming the ion channels. However, these data taken together with the binding data support a model in which the channel-forming portion of the protein is localized in a complex of a 76-kDa peptide fragment which is the part of the protein between amino acid 4476 and the carboxyl terminus of Ca\(^{2+}\) release channel. This same region contains both high and low affinity ryanodine binding sites (1) and part of the putative transmembrane domains of the Ca\(^{2+}\) release channel.

In summary, neomycin inhibits Ca\(^{2+}\) release and noncompetitively inhibits \(^{3}H\)ryanodine binding sites by an allosteric mechanism. The neomycin binding sites as well as the high and low affinity ryanodine binding sites are located in a peptide region encompassing the amino acid sequence from Arg-4475 to the carboxyl terminus.

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