Block by Ruthenium Red of Cloned Neuronal Voltage-Gated Calcium Channels

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ABSTRACT
The dye ruthenium red (RuR) has diverse experimental uses, including block of ion channels. RuR is well described as an antagonist of one class of intracellular Ca\(^{2+}\) release channels, the ryanodine receptors, but recently this compound has also been identified as a putative blocker of voltage-gated calcium channels of the surface membrane involved in neurotransmitter release. Using electrophysiological methods, we have studied the action of RuR upon pure populations of neuronal voltage-gated ion channels heterologously expressed in Xenopus laevis oo-
cytes. All four channel types studied, including class A (P/Q-type), class B (N-type), class C (L-type), and class E channels, are sensitive to RuR, with IC\(_{50}\) values ranging from 0.7 to 67.1 \(\mu\)M. Block of class C and class E channels most likely results from 1:1 binding of ruthenium red at a site in the extracellular entrance to the pore, resulting in obstruction of permeant ion flux through these channels. The mechanism of block of class A and class B channels is more complex, requiring binding of more than one molecule of RuR per channel.

Ruthenium red (RuR) is a water-soluble hexavalent cation with several major uses in biological research. The compound has been used as a stain in light microscopic studies of polyvalent anionic materials such as plant lectins, and RuR has been extensively used in combination with osmium tet-roxide as an electron-opaque dye for electron microscopic work. RuR is also an inhibitor of a surprisingly wide range of Ca\(^{2+}\)-binding proteins (Charuk et al., 1990). These include the mitochondrial Ca\(^{2+}\) uniporter (Moore, 1971), the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (Vale and Carvalho, 1973), troponin C (Charuk et al., 1990), calsequestrin (Char-
ruk et al., 1990), and calmodulin (Sasaki et al., 1992).

RuR is also well known as an antagonist of ryanodine receptors. These receptors are present in the intracellular membranes that bound internal Ca\(^{2+}\) stores and are ion channels responsible for signal-dependent release of stored Ca\(^{2+}\) into the cytosol. RuR binds directly to ryanodine recep-
tors (Smith et al., 1988; Chen and MacLennan, 1994) and blocks these channels by lodging in the pore (Ma, 1993). The apparent affinity for RuR block of ryanodine receptors is in the range of 0.1 to 1 \(\mu\)M. Sensitivity of Ca\(^{2+}\) release to RuR is one of the principal pharmacological means of distinguishing release via ryanodine receptors from that by 1,4,5-bis inositol trisphosphate receptors (Ehrlich et al., 1994).

Other kinds of ion channels are also affected by RuR.

Capsaicin-activated nonselective cation channels involved in nociception are inhibited by submicromolar concentrations of RuR (Dray et al., 1990; Bleakman et al., 1990). Inactivation of voltage-gated sodium channels is slowed by 1 to 10 nM RuR (Stimers and Byerly, 1982; Neumcke et al., 1987). Various actions on potassium channels have been described for micromolar RuR, depending upon channel type and whether RuR was applied extra- or intracellularly. Micromolar external (Hirano et al., 1998) or internal (Wann and Richards, 1994) RuR blocks large-conductance Ca\(^{2+}\)-activated potassium channels, internal RuR probably acting by antagonizing Ca\(^{2+}\) binding. RuR has also been reported to enhance the activity of other kinds of Ca\(^{2+}\)-activated potassium channels and of fast potassium channels while not affecting delayed rectifier type potassium channels (Lin and Lin-Shiau, 1996).

Like other Ca\(^{2+}\) binding proteins, including ryanodine receptors, the voltage-gated Ca\(^{2+}\) channels of cell surface membranes have been reported to be sensitive to RuR (Tapia et al., 1995; Tapia and Velasco, 1997). Voltage-gated Ca\(^{2+}\) channels in neurons from snail (Stimers and Byerly, 1982), rat (Hamilton and Lundy, 1995), and mouse (Lin and Lin-Shiau, 1996), and in smooth muscle of guinea pigs (Hirano et al., 1998) have all been reported to be blocked by RuR. In smooth muscle, the principal voltage-gated Ca\(^{2+}\) channel is an L-type channel sensitive to dihydropyridines, and this is presumably the channel blocked by RuR. However, in neu-
rons it has been reported that N- and P/Q-type Ca\(^{2+}\) channels, but not L-type Ca\(^{2+}\) channels, are antagonized by RuR (Hamilton and Lundy, 1995).

ABBREVIATIONS: RuR, ruthenium red.
These effects of RuR upon ion channels, particularly voltage-gated Ca\(^{2+}\) channels, have naturally consequences for synaptic transmission. For example, it has previously been shown that RuR interferes with normal neurotransmission (Taipale et al., 1989) and it is believed that RuR block of N- and P/Q-type Ca\(^{2+}\) channels is at least partly responsible for RuR inhibition of neurotransmission (Taipale et al., 1985; Hamilton and Lundy, 1995). RuR has also been reported to block a form of non-N-methyl-d-aspartate receptor-dependent synaptic plasticity (Wang et al., 1996b), an effect that may be attributable to Ca\(^{2+}\) channel block, ryanodine receptor block, or a direct action of RuR upon the neurotransmitter release apparatus (Trudeau et al., 1996).

Clearly, the spectrum of RuR action is broad, which in some cases leads to uncertainty in attributing the effects of RuR to specific molecular targets. To clarify the role of RuR as a putative antagonist of neuronal voltage-gated Ca\(^{2+}\) channels, we have measured the sensitivity to RuR of pure populations of neuronal (classes A, B, C, and E) voltage-gated Ca\(^{2+}\) channels heterologously expressed in Xenopus laevis oocytes, and we have investigated the mechanism of the antagonism. Each of these four channel types was blocked by RuR, but with differing half-block (IC\(_{50}\) values). The mechanism of block is in general complex, but involves at least in part binding within the channel entrance and obstruction of current flow.

**Materials and Methods**

**Expression of Ca\(^{2+}\) Channels in Xenopus Oocytes.** The methods are modified from Methfessel et al. (1986) and from Sather et al. (1993). Briefly, female Xenopus laevis were anesthetized by ~30-min immersion in a 0.2% tricaine methanesulfonate solution. The anesthetized frogs were placed on a bed of ice for the surgical procedure, with a layer of damp paper towels protecting the frogs' skin from ice burns. Ovarian tissue was removed via an abdominal incision, the incision was sutured, and the frogs returned to their housing for re-use in later experiments.

Individual oocytes were dissociated from the ovarian tissue by shaking for 90 min in Ca\(^{2+}\)-free OR-2 solution (in mM: 82.5 NaCl, 2 KCl, 1 MgCl\(_2\), 5 HEPES, pH 7.5 with NaOH) containing 2 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN). The oocytes were rinsed free of cellular debris and collagenase solution, and then selected oocytes were injected with cRNA encoding Ca\(^{2+}\) channel subunits.

Complementary RNAs encoding the various Ca\(^{2+}\) channel subunits were synthesized by in vitro transcription using SP6 or T7 polymerase and the following recombinant plasmids: pSPCB1-2 (rabbit a\(_{1C}\); Mori et al., 1991), pSPCBII (rabbit a\(_{1P}\); Fujita et al., 1993), pCARD3 (rabbit cardiac a\(_{1C}\); a\(_{1C}\);a\(_{1C}\); Mikami et al., 1989), pSPCBII-2 (rabbit a\(_{1P}\); Niidome et al., 1992), pSPCA1 (rabbit skeletal muscle a\(_{1}\);a\(_{1}\);a\(_{1}\); Mikami et al., 1989), and pCaB2b (rabbit b\(_{2b}\); Hullin et al., 1992). cRNAs were injected in a 1:1.1 M ratio, with a total volume of ~50 nl of cRNA injected into each oocyte. Injected oocytes were stored at 18\(^\circ\)C for up to 4 weeks in ND\(_6\) (in mM: 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, pH 7.6 with NaOH) supplemented with 2.5 mM sodium pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

**Two-Electrode Voltage Clamp Recording.** Whole cell Ca\(^{2+}\) channel currents were recorded using a standard two-electrode voltage clamp amplifier (model OC-725C; Warner Instruments, Hamden, CT). Glass microelectrodes typically had resistances of 0.3 M\(\Omega\) and were filled with 3 M KCl. Membrane currents were filtered at 0.5 KHz and sampled at 1 KHz. Leak and capacitive currents were subtracted using a P/4 protocol. Nevertheless, capacitive currents were incompletely subtracted, owing to the intrinsic slowness in clamping such large cells; the residual capacitative transients have been deleted from the figures. Depolarizing voltage pulses of 150-ms duration were delivered every 15 s during the experiments. The extracellular solution was chloride-free and contained (in mM): (nominally) 40 Ba(OH)\(_2\), 52 tetraethylammonium hydroxide, 5 HEPES, pH 7.4 using methane sulfonic acid. Addition of methane sulfonic acid to adjust solution pH to 7.4 caused significant precipitation of Ba\(^{2+}\); this precipitate was removed by filtration. Posthoc elemental analysis (Evergreen Analytical, Wheat Ridge, CO) of the nominally 40 mM Ba\(^{2+}\) solution revealed that the Ba\(^{2+}\) concentration ranged from 8.7 to 11.7 mM; in keeping with the extensive body of previous work utilizing this solution, however, we have continued to refer to this solution as “40 mM Ba\(^{2+}\)” throughout. A set of ten reservoirs, containing control solution or control solution supplemented with various concentrations of RuR, delivered a constant flow (~1 ml/min) through the slot-shaped recording chamber (15-mm length × 3-mm width × 3-mm depth).

The stoichiometries and apparent affinities of RuR action upon Ca\(^{2+}\) channels were determined by fitting dose-inhibition data with the Hill equation: \(I/I_{\text{max}} = 1/(1 + ([\text{RuR}]IC_{50})^m)\), where [RuR] is the concentration of RuR, IC\(_{50}\) is the half blocking concentration and represents an estimate of the apparent binding affinity, n\(_H\) is the Hill coefficient and provides an estimate of the functional stoichiometry, I is the measured current at any concentration of RuR, and I\(_{\text{max}}\) is the current measured in the absence of RuR. To obtain estimates of stoichiometry and apparent affinity for complex dose-inhibition relationships, sums of Hill terms similar in form to that described above were fit to the data.

Activation and inactivation parameters were obtained by fitting Boltzmann functions to normalized peak chord conductance data. The form of the equation for activation was G/G\(_{\text{max}}\) = \(1 + \exp(V_m - V_{\text{1/2}})^{-1}\), whereas that for inactivation was: G/G\(_{\text{max}}\) = \(1 + \exp(V_m - V_{\text{1/2}})^{-1}\), where V\(_{\text{1/2}}\) is the membrane potential, V\(_m\) is the midpoint voltages of the activation and inactivation functions, and b\(_a\) and b\(_i\) are the Boltzmann slope parameters for activation and inactivation.

The fraction of the electric field traversed by RuR in reaching its binding site was calculated using an equation of the following standard form: f\(_b\) = \([1 + (K_{\text{Des}}/\text{[RuR]}) \cdot \exp(-2zF(V_m/T))^{-1}\], where f\(_b\) is the fractional block, K\(_{\text{Des}}\) is the apparent dissociation constant measured at 0 mV, [RuR] is the concentration of the blocker, z is the valence of RuR (+6), \(\delta\) is the fractional electric distance from the outside of the membrane, F is the Faraday, R is the gas constant, T is the absolute temperature in Kelvin, and V\(_m\) is the test membrane potential. Because the apparent dissociation constant for RuR was measured at +20 mV (taken as the IC\(_{50}\)), the K\(_{\text{Des}}\) must be used; this is accounted for in calculating the electric distance by subtracting 20 mV from V\(_m\) inside the exponential term. The final equation used was: f\(_b\) = \([1 + (K_{\text{Des}}/\text{[RuR]})] \cdot \exp(-2zF(V_m - 20)/T/R))^{-1}\).

**Single Channel Recording.** For recording single class C channel activity, the oocyte vitelline membrane was removed by first shrinking the cell in a hyposmotic solution and then stripping the vitelline membrane away using forceps. Stripped oocytes in the recording chamber were bathed in a depolarizing solution (in mM: 100 KCl, 10 HEPES, 10 EGTA, pH 7.4 with KOH) to clamp the intracellular potential to 0 mV. Patch pipets were pulled from borosilicate glass, coated with Sylgard (Dow Corning, Midland, MI), and heat-polished to a resistance of ~18 to 25 M\(\Omega\) when filled with the 110 mM Ba\(^{2+}\) recording solution (in mM: 110 BaCl\(_2\), 10 HEPES, pH 7.4). In contrast to the 40 mM Ba\(^{2+}\) solution, there was no Ba\(^{2+}\) precipitation in the 110 mM Ba\(^{2+}\) solution. Cell-attached patches with seal resistances of typically ~20 to 100 G\(\Omega\) were obtained, and single channel currents recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at a corner frequency of 2 or 5 KHz (8-pole Bessel filter, Frequency Devices, Haverhill, MA), and sampled at 10 or 25 KHz using Pulse (Instrutech Corp., Great Neck, NY) software. The internal filter of the Axopatch 200B amplifier was set at 10 KHz in our experiments, so the effective corner
frequency of the cascaded filters was either 1.96 or 4.47 KHz. All single channel currents were inward currents carried by Ba$^{2+}$. In the single channel block experiments, RuR was included in the 110 mM Ba$^{2+}$ pipet solution. The trans-patch membrane potentials described are conventional for cell-attached patch recording, intracellular potential minus pipet potential. A nondihydropyridine agonist, FPL 64176 (2 μM, RBI, Natick MA), was included in the bath solutions to prolong channel open times and thereby facilitate the investigation of putative open-channel pore block. Analysis of single channel kinetics was carried out as described previously (Lansman et al., 1986).

RuR was obtained from either Fluka (Ronkonkoma, NY) or Sigma (St. Louis, MO). RuR action upon Ca$^{2+}$ channels was not different between these two sources, in agreement with previous findings that RuR obtained from various sources is essentially pure, regardless of dye content (Keohrer and Park, 1991). Because block by RuR was attenuated upon prolonged exposure of RuR-containing solutions to light, RuR solutions were protected from light and made fresh daily. All other chemical reagents were obtained from Fluka, Sigma, or Aldrich Chemical Company (Milwaukee, WI). All experiments were conducted at room (−22°C) temperature.

All mean values are reported together with S.E.M. Theoretical relationships (Hill equation, Boltzmann functions, and voltage-dependence of block) were fit to the data using a nonlinear least-squares routine (Marquardt-Levenberg algorithm) in SigmaPlot (Jandel Scientific, San Rafael, CA).

Results

Concentration Dependence of RuR Action. To estimate the binding affinity of RuR for various voltage-gated Ca$^{2+}$ channels, we studied the concentration dependence of RuR block of these channels. Superimposed records in Fig. 1 illustrate the action of RuR on class A, B, C, and E Ca$^{2+}$ channels. Three micromolar RuR blocked all four of the channel types we tested, but to differing degrees. Class A and B channels were most sensitive to block by RuR, class C channels were intermediate in sensitivity, and class E channels were least affected by 3 μM RuR. Complete dose-inhibition relations for block by RuR of these four classes of voltage-gated Ca$^{2+}$ channels are presented in Fig. 2. Block of class C and E channels was well-fit by 1:1 binding functions, yielding IC$_{50}$ values of 25.4 and 67.1 μM, respectively. The dose-inhibition relationships for class A and B channels were very similar to one another, and complex: one component (fraction = 0.75) of RuR block was described by a Hill coefficient of n$_H$ = −2 and an IC$_{50}$ of 0.7 μM, and a second component (fraction = 0.25) could be well-fit using a Hill coefficient of n$_H$ = −1 and an IC$_{50}$ of 25.4 μM. Thus, class A and B channels can be blocked by RuR in two distinct ways, whereas class C and E channels possess a single binding site for RuR. Interestingly, the lower affinity component of block of either class A or B channels was not distinguishably different from block of class C channels.

In these two-electrode voltage clamp experiments, the rate of development of block by RuR was obscured by the slowness of the solution exchange time and was therefore not studied. The recovery from block by RuR was very slow, but reversible. Because current amplitude ran down in a variable way over the time course of our experiments (30–60 min), a reliable unblock rate could not be obtained, but crudely, the block reversed with a time constant on the order of ~10 min.

Voltage Dependence of RuR Action. To further characterize the mechanism of RuR block of voltage-gated Ca$^{2+}$ channels, we examined the voltage dependence of RuR block in an attempt to determine whether RuR blocks by binding in the channel pore, or alternatively, if block results from modification by RuR of channel gating. The effect of RuR upon current-voltage relationships for each of the four Ca$^{2+}$ channel types tested is illustrated in Fig. 3A. Using doses of RuR that blocked between one- and two-thirds of the Ba$^{2+}$ current, we observed no significant change in reversal potential for any of the four channel types studied (see Legend for mean values). Furthermore, block by subsaturating doses of RuR had little effect on the shape of the current-voltage relations, for either the inward current carried by Ba$^{2+}$ or the outward current carried by K$^+$. Figure 3B plots fractional block of peak Ba$^{2+}$ current versus membrane potential, and shows that there was little voltage dependence to RuR block of class A, B, or C channels, but that block of class E channels was mildly, but clearly, voltage-dependent.

The voltage dependence of block of class E channels might arise either from RuR action on channel gating or by RuR binding within the channel's pore. If the voltage dependence of RuR block of class E channels arises from movement of this polyvalent cation partially into the electric field of the membrane during block, it is possible to calculate the product of
the effective charge of RuR (z) and the electrical distance from the outside of the membrane to the putative RuR binding site (δ). The value of zδ was estimated by fitting a modified form of the Boltzmann equation (see Materials and Methods) to the data for class E channels in Fig. 3B (dashed line), yielding a zδ of 0.36. Based on this potential mechanism of RuR block, and assuming that z = +6, the binding site for the blocker is very superficial (δ = 0.06), located only 6% of the way across the membrane electric field. However, RuR has a linear structure of \((\text{NH}_3)_b\text{Ru-O-Ru( NH}_3)_b\text{O-Ru(NH}_3)_b\) with the positive charge distributed evenly across the Ru(NH\(_3\))\(_n\) moieties. This extended structure is not expected to allow the entire molecule to penetrate the electric field, so the effective charge of the ion at the binding site is likely to be less than +6, which in turn would place the binding site deeper in the electric field.

RuR might alternatively or additionally affect the gating of Ca\(^{2+}\) channels. We therefore measured the voltage dependence of channel activation for class A, B, C, and E channels in zero RuR and in approximately half-blocking concentrations of RuR (Fig. 4A). Activation functions were calculated from normalized conductance values obtained using a range of test potentials because the slowness of the oocyte clamp precludes accurate measurement of tail currents. The absence of significant voltage dependence to RuR block of class A, B, or C Ca\(^{2+}\) channels is reflected in a similar lack of effect of RuR on activation functions for these three channel types (Fig. 4A). Likewise, the mild voltage dependence of RuR block of class E channels is reflected in the small positive shift in the apparent activation function for this channel type. A positive (right) shift in the activation function is consistent with the decrease in fractional block observed for increasingly positive test voltages.

We also investigated whether RuR had any effect on channel inactivation. Steady-state inactivation was measured by stepping the membrane potential from a variable amplitude prepulse (duration: 10 s) to a constant test voltage, for both zero RuR and in the presence of an approximately half-blocking concentration of RuR. As illustrated in Fig. 4B, RuR had little or no effect upon the steady-state inactivation behavior of any of the four channel types studied. Nor did RuR alter the kinetics of inactivation for any of the four channel types as evidenced by the fact that fractionally blocked currents can be seen, when normalized and superimposed, to follow the same time course as the unblocked currents (Fig. 5). The speed of the voltage clamp prevented high-resolution analysis of the activation kinetics, but nonetheless, it is clear that RuR does not profoundly slow activation time course.

**Block of Closed Channels by RuR.** Can RuR block channels even when the channel is not open? To help address this issue, we examined whether RuR can block closed channels as well as it can block open channels. Experiments such as that illustrated in Fig. 6 were specifically designed to test whether RuR block of nonactivated (unused) channels develops as rapidly as when channels are regularly activated (used). In one set of experiments, after establishment of a steady amplitude for peak inward Ba\(^{2+}\) currents, RuR was applied and the time course of block measured. In a separate set of experiments, after establishment of a steady amplitude for peak inward Ba\(^{2+}\) currents, the test depolarizations were stopped and RuR was applied at the same time. After waiting...
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Fig. 5. Examples of normalized current records from individual cells expressing classes A, B, C, or E Ca\textsuperscript{2+} channels before and during 1 \mu M (class A, B), 10 \mu M (class C), or 100 \mu M (class E) RuR treatment. Example records of currents measured in RuR have been normalized and superimposed upon records of currents measured in zero RuR. RuR inhibited peak Ba\textsuperscript{2+} current by approximately 50% in each cell. Currents were recorded with a holding potential of ~80 mV and test potential of +20 mV.

Fig. 6. RuR blocks closed channels as well as open channels. The average time course of block by RuR (n = 3 cells) when test pulses were applied every 15 s is superimposed with a representative plot of block by RuR when test pulses were stopped for 180 s (class A, B, and C) or 360 s (class E), and then resumed with RuR still in the bath. The time courses are aligned in time so that the start of the RuR applications coincide. Currents were recorded with a holding potential of ~80 mV and test potential of +20 mV. 10 \mu M (class A, B) or 100 \mu M (class C, E) RuR was applied.

3 to 6 min, the constant amplitude test depolarizations were restarted, allowing measurement of Ba\textsuperscript{2+} currents and their fractional block. Comparisons were made between different cells because the slow reversibility of RuR block did not allow the two experimental protocols to be carried out on the same oocyte.

For class A, B, C, and E channels, block developed during the period when the channels were unused, to the extent that by the end of the 3- to 6-min waiting period, block had already reached a nearly steady-state level. Once the test depolarizations were reinitiated, channels were revealed to be blocked to the same degree as those recorded from oocytes for which test depolarizations were not halted. Comparison of the superimposed time courses for fractional block (Fig. 6) suggests that block developed at approximately the same rate, whether channels were used or not, and certainly that block did not develop more slowly for channels that were not used. Thus block of Ca\textsuperscript{2+} channels by RuR is not use-dependent.

Block of Single Open Channels by RuR. In attempting to determine whether RuR block of voltage-gated Ca\textsuperscript{2+} channels occurs fundamentally by modifying gating, or alternatively, by blocking the channel pore, we examined the action of RuR upon single Ca\textsuperscript{2+} channels. In these experiments, we studied the class C channel only, because openings can be prolonged in this channel type by the use of a channel agonist such as FPL 64176. Extended channel open duration is useful because it facilitates resolution of channel-blocking events.

Comparing single channel openings in the absence of RuR with those obtained in the presence of RuR reveals that the blocker causes brief interruptions in the flow of current through the class C channels (Fig. 7A). The longer shut times between the bursts of open/shut transitions correspond to the normal closed time of the channel in the absence of RuR. The number of brief blocking events increased with dose of RuR, such that the time-integrated current carried by an open channel is reduced by RuR. RuR solutions exposed to light produced fewer flicker block events, consistent with an interpretation of reduced block resulting from photodegradation of RuR dye (data not shown; see Materials and Methods).

Analysis of the kinetics of RuR block of class C channels is presented in Fig. 7B. RuR block follows bimolecular kinetics with a concentration-dependent association rate and concentration-independent dissociation rate. The apparent association rate (1.7 \times 10^7 M^{-1} s^{-1}) is an order of magnitude below the diffusion-limited encounter rate predicted for a molecule the size of RuR, whereas the apparent dissociation rate (5849 s^{-1}) predicts that class C channels would recover from block in less than 0.2 ms. The fast unblock of single channels is in sharp contrast to the very slow unblock observed using two-electrode voltage-clamp measurements. It is possible that the slow recovery from block in two-electrode voltage-clamp experiments arose from adsorption of RuR onto the oocyte membrane and that slow unblock in those experiments in fact reflected slow removal of RuR from the oocyte (Voelker and Smejtek, 1996). From single-channel kinetic analysis, the apparent dissociation constant (k_{dissociation}/k_{association}) is 344 \mu M, or about 13-fold larger than the IC\textsubscript{50} determined from the dose-inhibition data (Fig. 2). Assuming that RuR

Fig. 7. Action of RuR on single class C Ca\textsuperscript{2+} channels in cell-attached patches. A, example records of single channel currents recorded in 0, 10, 30, and 65 \mu M RuR. In these experiments, the membrane potential was stepped from the holding potential (~80 mV) to a prepulse value of +40 mV to activate channels with high probability, and then the patch was stepped down to ~20 mV to facilitate study of RuR block by increasing the amplitude of the single channel currents. RuR was included in the pipet solution (110 mM Ba\textsuperscript{2+}) at the indicated concentrations, and 2 \mu M FPL 64176 was present in the bath solution to prolong channel openings. B, rates of block (reciprocal of mean open time) and unblock (reciprocal of mean closed time during a burst) as a function of RuR concentration. Three patches were analyzed at each concentration of RuR, and the mean and S.E.M. values plotted. All data in part B were filtered at 4.47 KHz and sampled at 25 KHz. The block rate was 1.7 \times 10^7 M^{-1} s^{-1}, the unblock rate was 5849 s^{-1}, and the apparent dissociation constant was 344 \mu M.
must compete with $\text{Ba}^{2+}$ for entry into the channel mouth, the discrepancy in apparent binding affinity is readily attributable to the approximately 11-fold difference in $\text{Ba}^{2+}$ concentration (see Materials and Methods) used in the two kinds of experiments.

**Discussion**

RuR has a long and extensive history of use in many experimental settings, including a well known role as an antagonist of one class of intracellular $\text{Ca}^{2+}$-release channels, the ryanodine receptors. RuR has also been identified as a likely blocker of voltage-gated $\text{Ca}^{2+}$ channels in the cell surface membrane (Stimers and Byerly, 1982; Tapia et al., 1985; Hamilton and Lundy, 1995; Lin and Lin-Shiau, 1996; Tapia and Velasco, 1997; Hirano et al., 1998). However, direct observation of RuR block of mammalian neuronal $\text{Ca}^{2+}$ channel currents has not been described previously. Furthermore, there are several major subtypes of voltage-gated $\text{Ca}^{2+}$ channels in mammalian neuronal surface membranes, including L-, N-, and P/Q-type channels, and the possible selectivity of RuR block of the various subtypes has previously been unclear. Here we have shown, using essentially pure populations of heterologously expressed channels, that RuR blocks several kinds of mammalian voltage-gated $\text{Ca}^{2+}$ channels with half-block concentrations in the range of those described previously for $\text{Ca}^{2+}$ channels in native tissues. In contrast with some earlier reports, we have found that L-type channels as well as non-L-type channels are blocked by RuR. The results of our experiments also help to elucidate the mechanism of RuR action upon these channels.

**RuR Sensitivity of Distinct Channel Types.** Both dihydropyridine-sensitive L-type $\text{Ca}^{2+}$ channels (class C) and non-L-type channels (classes A, B, E) are sensitive to RuR. Previous studies, lacking the advantage of using nearly pure populations of channels, had come to conflicting conclusions in this regard, some suggesting that L-type channels are not sensitive to RuR. Part of the explanation for the discrepancy may be that class A and B channels are more sensitive to RuR than are class C channels. The results of our dose-inhibition measurements also address the possibility, raised by others in earlier work, that RuR might prove to be an inexpensive and readily available antagonist for particular $\text{Ca}^{2+}$ channel subtypes. Based on our results, the differential sensitivity to RuR between channels involved in neurotransmission (classes A and B) and those that play other roles in cells (for example, class C), may be exploitable in a limited way, but the dose-inhibition relationships are not widely separated, which ultimately disfavors use of RuR as a means to discriminate among $\text{Ca}^{2+}$ channel subtypes in cells possessing mixed populations of these channels.

Earlier work focused on functionally defined channel types present in native tissues, particularly P/Q-type (class A), N-type (class B), and L-type (class C) channels. Class E channels do not have a clear functional correlate, although they are often linked to “R-type” channels. Our characterization of RuR action on class E channels therefore represents the first description of such action. Class E channels were the least sensitive to RuR of the four channel types we tested. Class A, B, and E channels are closely related to one another and are structurally divergent from the non-L channels, including class C channels. Block of class A channels is virtually identical with block of class B channels, both in the multi-component nature of the dose-inhibition relationships and in the IC$_{50}$ values. Why class E channels are different from the two other non-L-type channels, being less sensitive to RuR than are class C channels, is not clear. Inspection of pore-lining sequences, particularly in regions known to form the pore entrance, reveal differences in amino acid sequences, but little that could obviously account for the observed differences in RuR binding.

**Mechanism of RuR Block: Number of Sites.** Block of class C and E channels is well described by binding of a single molecule of RuR per channel. The RuR binding site differs slightly in affinity between these two channel types, based on IC$_{50}$ values of 25.4 $\mu$M for class C channels and 67.1 $\mu$M for class E channels. The difference in affinities translates to a free energy difference in the binding of RuR to these sites of about 0.6 kcal/mol, which is roughly one-tenth of the molar energy of hydrogen bonding.

Class A and B channels are very similar to one another in their block by RuR, but unlike C and E channels, there are two components to block of A and B channels. In our experiments $>95\%$ of the $\text{Ba}^{2+}$ current is carried by the heterologously expressed channels, so a contaminating contribution of endogenous oocyte $\text{Ca}^{2+}$ channels to the dose-inhibition relationships cannot account for the complex form of the data. The lower-affinity component, which comprises approximately 25% of block, can be described by the binding of one RuR molecule per channel. To the extent that stoichiometries and affinities are similar, the lower-affinity site on A and B channels may correspond to the single site of C and E channels. The higher-affinity component of block of A and B channels, which comprises approximately 75% of the total block of these channels by RuR, appears to involve the binding of two RuR molecules per channel (Hill coefficient is $-2$). Full block by RuR of A or B channels may require the binding of as many as three RuR molecules.

Comparison of RuR block of A and B channels to RuR block of ryanodine receptors is useful. Hill slopes for block of ryanodine receptors by RuR are $\approx 2$, the interpretation in that case being that two or more RuR molecules bind within the pore of ryanodine receptors (Ma, 1993). If a similar process occurs in A and B channels, then an additional assumption must be made: even when the high-affinity sites are fully occupied, the channel is not completely blocked ($\approx 75\%$). Alternatively, this component of block may result from alteration by RuR of channel gating.

**Mechanism of RuR Block: Pore Blocker Versus Gating Modifier.** We have considered two mechanisms for RuR block of voltage-gated $\text{Ca}^{2+}$ channels: physical obstruction of the pore or a shift in the voltage-dependent probability of channel opening. Other mechanisms of block are less likely. Screening of membrane surface charge by RuR is not the exclusive mechanism of block because RuR blocks single channel currents in high ionic strength solutions (110 mM $\text{Ba}^{2+}$; Kuo and Hess, 1992; Block et al., 1998). Loss of channels into a long-lived nonconductive state is unlikely because single channels open very frequently even in the presence of high concentrations (1 mM) of RuR.

The sum of the data suggests, but does not conclusively show, that block by RuR results from binding to a superficial site in the extracellular entrance to the pore of the channel. There is little or no voltage dependence to RuR block, indi-
cating that the site is essentially outside of the membrane electric field. That RuR blocks closed channels as well as open channels (Fig. 6) is also consistent with an easily accessed site at the entrance to the pore.

The evidence most suggestive of a pore block mechanism is the observation of transient, RuR-generated interruptions of current flow through activated single channels, with the number of block events increasing in a dose-dependent manner. The bimolecular nature of the block kinetics and the values of the rate constants of block and unblock are highly reminiscent of other small pore blockers, such as Cd²⁺. In addition, the near absence of effect of RuR upon steady-state activation or inactivation suggests that at least for most channel types, RuR does not act by altering the voltage dependence of channel gating.

Nevertheless, the alternative that block by RuR might derive from modification of channel gating is worth considering. According to this hypothesis, the brief interruptions of current flow through open channels observed in the presence of RuR represent allosteric RuR-induced channel closures. Assuming that the gating modification hypothesis is correct, RuR not only speeds the closing rate, but it must also speed the reopening rate because the current interruptions are very brief (Fig. 7). In the presence of RuR, there are two distinct opening rates, one within a burst (designated “opening”), and one between bursts (designated “opening”). The latter rate is small (kₜ₉₉ = 1/interburst lifetime) < 100 s⁻¹ and corresponds to the normal, slow opening rate in the absence of RuR. The fast reopening rate describes the return to the open state from the brief, RuR-induced shut events (kₜ₀ₐₜ = 1/brief shut lifetime) ~ 1000 s⁻¹. Such a mechanism is complex and consequently somewhat less plausible than the relatively straightforward pore block hypothesis.

An experimental paradigm that would be helpful in distinguishing between the two block mechanisms involves comparison of RuR block for two different concentrations of permeant ion on the opposite (intracellular) side of the membrane. Testing for “knock-off” of RuR by an internal permeant ion was not possible because Ca²⁺ channels do not often survive patch excision, and our attempts to borrow a strategy of elevating intracellular Ca²⁺, Mg²⁺, and ru-RuR upon steady-state activation or inactivation suggests that at least for most channel types, RuR does not act by altering the voltage dependence of channel gating.

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