Slowing of Sodium Current Inactivation by Ruthenium Red in Snail Neurons

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ABSTRACT The effects of ruthenium red (RuR) were tested on the membrane currents of internally perfused, voltage-clamped nerve cell bodies from the snail Limnea stagnalis. Bath application of nanomolar concentrations of RuR produces a prolonged Na current that decays ~40 times slower than the normal Na current in these cells. The relationship between the reversal potential for the prolonged Na current and the intracellular concentration of Na agrees well with the constant-field equation, assuming a small permeability for Cs. Because a strong correlation was found between the magnitude of the normal Na current and that of the prolonged Na current, it is concluded that the prolonged Na current flows through the normal Na channels. This conclusion is supported by the similar selectivities, voltage dependencies, and tetrodotoxin (TTX) sensitivities of these two currents. This action of RuR to slow the inactivation of the Na channel was not observed at concentrations below 1 nM, but was complete at 10 nM. When the concentration of RuR is increased to 0.1 mM, the Ca current in these cells is blocked; but at this high concentration RuR also reduces the outward voltage-dependent currents and resting membrane resistance. Therefore, RuR is not a good Ca blocker because of its lack of specificity. However, its action of slowing Na current inactivation is very specific and could prove to be useful in studying the inactivation of the Na channel.

INTRODUCTION

It has been suggested that ruthenium red (RuR) blocks Ca channels in synaptosomes (Swanson et al., 1975; Tapia and Meza-Ruiz, 1977) and the frog neuromuscular junction (Alnes and Rahamimoff, 1975; Rahamimoff and Alnes, 1973; Person and Kuhn, 1979). The voltage-dependent and spontaneous transmitter release from the frog neuromuscular junction is blocked by RuR (Person and Kuhn, 1979). RuR also blocks release from rat brain synaptosomes (Tapia and Meza-Ruiz, 1977). Furthermore, Ca uptake into synaptosomes is blocked by RuR (Swanson et al., 1975). This suggestion was recently called into question when Baux et al. (1978, 1979) found that RuR did not block Ca spikes in Aplysia neurons. However, they did find that synaptic transmission was blocked by RuR. They suggested that RuR occupies the intracellular Ca²⁺ binding sites, which must bind Ca²⁺ for transmitter release to occur.
Because of our general interest in drugs that act specifically on Ca channels and an interest in the use of RuR for neurophysiological studies, we have investigated the effects of RuR on the voltage-dependent currents in internally perfused snail neuron cell bodies. This preparation is especially suitable for this study because of its advantages for voltage-clamp analysis. Because these cells are completely isolated from the rest of the nervous system and are nearly spherical, good spatial and temporal control of the membrane potential is achieved under voltage clamp. Complicated spatial geometries usually interfere with this goal. Voltage-clamp analysis allows one to accurately determine the effects of RuR on membrane currents. We found that high concentrations of RuR (>0.1 mM) do block the Ca current; however, this effect is not very specific. The same concentrations of RuR also reduce the voltage-dependent K currents and the nonspecific current (Byerly and Hagiwara, 1982), as well as decreasing the resting membrane resistance. The most specific action of RuR is on the Na channel, the inactivation of which is greatly slowed in the presence of very low levels of RuR (10 nM). Preliminary results of these experiments have been published (Byerly and Stimers, 1981).

**METHODS**

Trypsin-treated (0.2% for 105 min at room temperature), isolated neuron cell bodies from the snail *Lymnea stagnalis* were internally perfused and voltage clamped according to the methods of Byerly and Hagiwara (1982). This suction-electrode, voltage-clamp technique, which is applied to neuron cell bodies that have been previously isolated from the rest of the nervous system, allows the free exchange of intracellular ions with the perfusing solution. The suction pipette was used both to record membrane potential and to inject current. As described in Byerly and Hagiwara (1982), this allows a clamp of the membrane potential such that the capacitive currents settle in 0.5 ms and the error in the measured voltage is <1 mV for currents up to 30 nA.

**Solutions**

Table I shows the composition of the intracellular and extracellular solutions used. In the extracellular solutions, 0.1 mM Cd^{2+} and 10 mM 4-aminopyridine (4-AP) were added to the desired solution without lowering the concentration of any other ions. In these experiments, 0.1 mM Cd^{2+} was used to block the Ca current. This concentration
of Cd\(^{2+}\) blocked >90% of the Ca current. Effects of Cd\(^{2+}\) on the Na current and surface potential were minimal at this concentration, but became significant if the Cd\(^{2+}\) concentration was increased sufficiently (to 1 mM) to obtain a complete block of the Ca current. This is not unexpected because Ca channel blockers are known to partially block the Na current (Hagiwara and Byerly, 1981). The presence of 4-AP in the external solution reduces the nonspecific current in these cells (Byerly and Hagiwara, 1982). Internal and external solutions were adjusted to pH 7.3 and 7.4, respectively. All experiments were done at room temperature.

**Ruthenium Red**

Ruthenium red ([\((\text{NH}_3)_5\text{Ru-O-Ru(\text{NH}_3)}_4\text{-O-Ru(\text{NH}_3)}_5\)Cl\(_6\)]) is an inorganic molecule that has up to six positive charges when in solution. The RuR (Sigma Chemical Co., St. Louis, MO) used in these experiments was not purified by us in any way. A molecular weight of 786.35 was used in all calculations of concentrations. Because the commercial RuR used here is \(\sim 50\%\) pure (the contaminating material is probably ruthenium violet), the actual concentration of RuR in each solution was \(\sim 50\%\) of that given in this paper.

**RESULTS**

**Action of RuR on the Na Current**

RuR has a striking effect on the time course of the current seen under conditions where only a fast-inactivating Na current was initially present. When a cell is bathed in 0-K Limnea saline plus 0.1 mM Cd\(^{2+}\) plus 10 mM 4-AP and internally perfused with Cs-aspartate, a fast-inactivating Na current is seen on stepping from the holding level (−50 mV) to potentials more positive than −30 mV. Fig. 1A shows examples of the fast-inactivating Na current under these conditions (upper current trace in each pair). Because only 90% of the Ca current in this cell was blocked by Cd\(^{2+}\), the residual Ca current shows up as a steady state current in the records. This fast-inactivating Na current is blocked by TTX (80% by 40 \(\mu\)M). Addition of 10 \(\mu\)M RuR to the bath changes the current evoked by a positive pulse to a prolonged inward current (Fig. 1A, lower trace of each pair). The magnitude of the peak current, before and after the addition of RuR, as a function of voltage is plotted in Fig. 1B. It is obvious from these curves that the voltage dependence of the two currents, the fast-inactivating Na current and the prolonged current, are nearly identical; there is a <5 mV shift between the two curves.

It is of interest to note that the time course of this prolonged current is nearly identical with that of the Ca current described in these cells (Byerly and Hagiwara, 1982). However, the prolonged inward current is abolished when Na\(^{+}\) is replaced with Tris, which suggests that this current is a prolonged Na current. The transition of the currents from a fast-inactivating Na current to a prolonged Na current is complete within 30 s after the addition of RuR to the bath. This fast action of RuR would suggest that its site of action is at the external surface of the membrane, especially when the multivalent nature of the molecule is considered.

The normal Na current at +10 mV decays to 50% of its peak value in \(\sim 1\)
ms. The voltage dependence of the time constant of decay for the normal Na current is shown in Fig. 2 (filled circles). The prolonged Na current requires 60 ms to decay to 50% of its peak value at +10 mV. As can be seen in the records of Fig. 1A, the prolonged inward current does not decay as a simple exponential function; this is caused, at least in part, by the activation of the nonspecific outward current. To make a comparison with the decay of the normal Na current, the prolonged Na current was assumed to decay as a single exponential, the time constant for which was calculated from the maximum value of the current and the value of the current at the end of the 60-ms pulse. Fig. 2 (open circles) shows the voltage dependence of these decay time constants for the prolonged Na current in RuR. In RuR, the inactivation of the Na current is ~40 times slower than normal, at potentials between −10 and +50 mV.

Selectivity of RuR-sensitive Conductance

As a step in identifying the action of RuR on membrane currents, it was necessary to establish that Na⁺ actually carries the prolonged inward current
induced by RuR and to determine the selectivity of this conductance for Na$^+$ over other alkaline cations. The disappearance of the prolonged inward current when external Na$^+$ is replaced by Tris$^+$ does not necessarily establish that Na$^+$ carries the prolonged current; for example, in the starfish egg membrane, a Ca current is abolished by removal of external Na$^+$ (Hagiwara et al., 1975). The selectivity of the RuR-sensitive conductance is of particular interest because Kostyuk and Krishtal (1977b) reported that EGTA in the absence of external divalent cations caused the appearance of a similar prolonged inward Na current. They suggested that this Na current was flowing through Ca channels.

To establish that Na$^+$ did pass through the RuR-sensitive conductance and to determine the selectivity of the conductance for Na$^+$ over Cs$^+$, the reversal

**Figure 2.** Voltage dependence of the time constant of inactivation. The data are from the same cell as in Fig. 1. Closed circles indicate the values without RuR and the open circles are with RuR. Time constants for the prolonged current (in RuR) were calculated from the maximum current and the value at the end of a 60-ms pulse, assuming an exponential decay. Each time constant was calculated assuming that the value of the current at 60 ms without RuR was the “zero current level” for both before and after the addition of RuR. A second calculation of the time constant was made using 50% of the value of the current at 60 ms without RuR as the “zero current level.” The range between these two values is plotted as the error bars and the points represent their average. This method was used as a control for the presence of any other currents. Because RuR was found to block both the Ca and nonspecific currents, we feel that this is a reasonable method to use.
potential for the RuR-induced current was measured with various concentrations of internal Na\(^+\), replacing Na\(^+\) with Cs\(^+\). Cells were bathed in 0-K *Limnea* saline plus 0.1 mM Cd\(^{2+}\) plus 10 mM 4-AP. The reversal potential was determined as the potential at which the I-V curve measured before RuR was added to the bath intersected the I-V curve measured after the application of RuR. Current values were taken 10 ms after the onset of the clamp pulse. The reversal potential obtained by this method agreed well with the values determined by two other methods: (a) the visual estimation of the potential at which the current trace was flat (Fig. 3, inset), and (b) the intersection of the I-V curve in RuR with a linear projection of the leak current measured in the hyperpolarizing direction. The data from this experiment are plotted in Fig. 3. Each point represents the reversal potential from a single cell. The solid line, a least-squares fit to the data, has a slope of 55 mV/decade. The dashed line is a plot of the constant-field equation:

\[
V_r = -59 \text{ mV} \log\left(\frac{[\text{Na}^+]_i + P[\text{Cs}^+]_i}{[\text{Na}^+]_o}\right)
\]

where \(P\) has the value 0.036 and is the permeability of the membrane to Cs\(^+\).
relative to that to Na+. The value of \( P \) was determined by measuring \( V \), when \([\text{Na}^+]_i = 0 \text{ mM}\). The slope of this curve is \(~54 \text{ mV/decade}\) in this region, which agrees well with the experimental line. This good agreement between the slopes of the experimental and theoretical relations shows that the prolonged inward current induced by RuR is carried by Na+ and that the permeability of this conductance to Cs+ is only 0.036 times the permeability to Na+. This relative permeability is very similar to that for the normal Na channel (Chandler and Meves, 1965; Hille, 1972; Okamoto et al., 1976; Takahashi and Yoshii, 1978).

The 10-mV offset between the experimental and theoretical lines is caused, at least in part, by a lower activity coefficient for Na+ in the internal solution than for external Na+, which is neglected in the above constant-field equation. The offset might also indicate a small permeability for one of the other external cations (Ca2+, Mg2+, or Tris+), although these ions carry no detectable inward current when external Na+ is removed. We are confident that the offset is not caused by an error in potential measurement or incomplete exchange of internal Na+. We have checked the potential measurement by recording with a microelectrode inserted through the membrane. Incomplete exchange is ruled out, because the cells are perfused with the desired internal solution for at least 10 min, although the exchange of intracellular Na+ is complete in 5 min, as measured by changes in membrane currents or recordings from intracellular K-sensitive microelectrodes (Byerly and Moody, 1982). This conclusion is also supported by the observation that the measured reversal potential is always more positive than the predicted value, independent of whether the cell was previously perfused with higher or lower concentrations of Na+.

Channel Carrying the Prolonged Na Current

To determine whether this prolonged Na current passes through either the normal Na or Ca channels, the magnitude of the fast inactivating Na current \((I_{Na})\), the Ca current \((I_{Ca})\), and the prolonged Na current \((I_{Na,L})\) induced by RuR were compared for 11 cells (Table II), 10 of which are plotted in Fig. 4. Cell 11 was not plotted because of the extraordinarily large Na currents. Each cell was bathed in 0-K Linnea saline and internally perfused with Cs-aspartate. The potential at which the maximum inward current at 10 ms occurred was determined. This maximum current was taken as \(I_{Ca}\) for that cell. Next, 0.1 mM Cd2+ was added to the bath to block the Ca current. Again, a voltage-clamp series was done to determine the maximum \(I_{Na}\). As shown in Fig. 4A, \(I_{Na}\) was measured as the peak inward current minus the current at 10 ms. Finally, 10 \(\mu\)M RuR was applied. The prolonged Na current \((I_{Na,L})\) was taken as the current after RuR was applied minus the current just before the addition of RuR (both were measured 10 ms after the pulse beginning).

Fig. 4 shows the correlation between these currents. As can be seen in Fig. 4B, there is very little correlation between \(I_{Na}\) and \(I_{Ca}\) (correlation coefficient of 0.05, including cell 11); most cells studied had a larger Ca than Na current. Fig. 4C shows that the Ca current is not correlated with the prolonged Na current (correlation coefficient of 0.14, including cell 11), whereas Fig. 4D
shows a strong correlation between the fast-inactivating and the prolonged Na currents (correlation coefficient of 0.99, including cell 11). These relations suggest that $I_{Na}$ and $I_{Na,L}$ are passing through the same channel. Cell 11 shows these relationships clearly with both $I_{Na}$ and $I_{Na,L}$ being very large, whereas $I_{Ca}$ is relatively small.

**TABLE II**

**CORRELATIONS BETWEEN $I_{Ca}$, $I_{Na}$, AND $I_{Na,L}$**

| Cell | $I_{Ca}$ | $I_{Na}$ | $I_{Na,L}$ |
|------|----------|----------|------------|
| 1    | 64       | 35       | 29         |
| 2    | 30       | 47       | 41         |
| 3    | 5.5      | 16       | 12         |
| 4    | 53       | 19       | 25         |
| 5    | 26       | 7.0      | 4.8        |
| 6    | 72       | 21       | 24         |
| 7    | 25       | 13       | 8.5        |
| 8    | 28       | 3.8      | 4.0        |
| 9    | 2.0      | 18       | 11         |
| 10   | 17       | 12       | 8.5        |
| 11   | 27       | 160      | 130        |

**FIGURE 4.** Relationships between $I_{Ca}$, $I_{Na}$, and $I_{Na,L}$. A. This drawing illustrates the method for determining the magnitudes of these three currents. The external solutions used are indicated on the current traces (0.1 mM Cd$^{2+}$ and 10 μM RuR were used; L.S. = Limnea saline). The internal perfusion solution was Cs-aspartate in all cases. See the text for further details. B–D. $I_{Ca}$, $I_{Na}$, and $I_{Na,L}$ are plotted here to illustrate the relationships that may exist between them.
Further evidence that $I_{Na,L}$ passes through the normal Na channel is provided by the blocking action of TTX. When the Na current is isolated by applying internal Cs$^+$ and external Cd$^{2+}$ to eliminate the K and Ca currents, respectively, we find that 40 μM TTX blocks 80% of the fast-inactivating Na current. Washing the TTX out of the bath restores the Na current. Adding RuR to the bath results in the prolonged Na current being induced. This current is also 80% blocked by 40 μM TTX in the bath. This evidence leaves little doubt that the prolonged Na current induced by RuR is passing through the normal Na channels and that the RuR is interfering with the inactivation process. Conflicting reports on the TTX sensitivity of these neurons have appeared in the literature. Kostyuk and Krishtal (1977a) found that 6 μM TTX had no effect on the Na current of snail neurons extensively treated with trypsin (1% for 60 min at 37°C). Lee and co-workers (1977) reported that a brief trypsintreatment (0.1% for 2–4 min at room temperature) made the Na channel resistant to even 150 μM TTX. However, Chemeris et al. (1980) demonstrated that the loss of TTX sensitivity produced by a mild trypsin treatment is reversed by further trypsin treatment. Thus, after intensive trypsin treatment, such as we use, the TTX sensitivity is intact.

**Action of RuR on the Ca Current**

Our original interest in RuR was to assess its effectiveness as a Ca-channel blocker. When a cell is internally perfused with Cs-aspartate and bathed in Tris saline, an inward Ca current is recorded when the membrane potential is stepped from the holding level (–50 mV) to potentials more positive than –20 mV (Byerly and Hagiwara, 1982). Under these conditions, 0.1 mM RuR added to the bath will block ~90% of the Ca current, which is roughly the same block produced by 0.1 mM Cd$^{2+}$. At this concentration of RuR, however, other effects of the dye are seen. Both the membrane resistance and the nonspecific outward current, which is voltage and time dependent (described in Byerly and Hagiwara, 1982), are reduced. When a cell is internally perfused with K-aspartate, ~50% of the K current is also blocked at these concentrations of RuR.

We do not believe that the block of the K current is secondary to the block of the Ca current. That is, the K current being blocked is not a Ca-dependent K current. When a cell is internally perfused with K-aspartate and bathed in Tris saline, the current recorded from a voltage-clamped cell has an early inward component and a late outward component, corresponding to the Ca and K currents, respectively. The relative magnitude of each component varies from cell to cell. When a cell that has no net inward current is exposed to 0.1 mM RuR, the K current is still reduced by nearly 50%. Because this cell possesses very little Ca current, it is reasonable to assume that the Ca-dependent K current is also small. In fact, the Ca-dependent K currents in all cells studied may be small, since the internal solution contains 5 mM EGTA. Therefore, it is likely that RuR is interacting with another type of K channel, probably the delayed rectifier.
Dose-Response Curves

When the effect of RuR dosage was examined, we found that the two actions of RuR, blocking the Ca current and slowing inactivation of the Na current, occur at very different levels. The dose-response curve for the action of RuR in slowing Na current inactivation is shown by the left-hand curve in Fig. 5. This relation is obtained by sequentially exposing each cell to increasing concentrations of RuR. As can be seen in the figure, the RuR effect on Na current inactivation is completely saturated at 10 nM RuR (half-maximum at 2.9 nM). This curve (drawn by eye) is too steep to represent one-to-one binding, and would suggest that at least three RuR molecules are required to produce the effect.

![Dose-response curves. The effects of RuR dosage on Na current inactivation (left) and on blocking of the Ca current (right) are shown.](image)

**Figure 5.** Dose-response curves. The effects of RuR dosage on Na current inactivation (left) and on blocking of the Ca current (right) are shown. Each cell was bathed in either 0-K *Limnea* saline + 0.1 mM Cd^{2+} + 10 mM 4-AP (for I_{Na,L}) or Tris saline (for I_{Ca}) and internally perfused with Cs-aspartate. The membrane potential was held at −50 mV and stepped to the level at which the current of interest was a maximum (near +10 mV for I_{Na,L} and +20 mV for I_{Ca}). The value of the current at 10 ms was recorded before the addition of RuR and at each concentration of RuR that was subsequently applied. The prolonged Na current was calculated as the magnitude of the inward current at each concentration of RuR minus that before RuR was added; normalized values are plotted. A solid curve is drawn by eye through this data. The magnitude of the Ca current was calculated as the value of the current in each concentration of RuR minus the leakage current measured with hyperpolarizing pulses. The dashed curve drawn through the data shows the relation expected for one-to-one binding. Different symbols represent data from different cells.

The dose-response curve for the block of the Ca current (Fig. 5, right) was obtained by a procedure that was similar to that used to obtain the Na channel dose-response curve. The blocking action of RuR on the Ca channel increases over a broad range of concentrations. The relation expected for one-to-one binding fits these dose-response data quite well (dashed curve), with a dissociation constant of 5.4 μM. Comparing the two dose-response curves of Fig. 5, one can see that the Na-channel effect saturates before the Ca-channel effect can be detected. This clearly shows that the action of RuR in producing the prolonged Na current is independent of the block of the Ca current.
DISCUSSION

RuR as a Ca-Channel Blocker

RuR does block the Ca channel present in the membrane of snail neuron cell bodies, with 50% block occurring at 5.4 µM RuR. However, RuR is not a good blocker for the Ca channel because it is not very specific. Concentrations of RuR that block the Ca channel also decrease the membrane resistance, partially block the K and nonspecific currents, and cause shifts in voltage dependence of, at least, the Na current.

Because this work was done with isolated cell bodies, it is not possible to state what effect RuR has on synaptic transmission in the snail. However, it is reasonable to assume that transmission would be blocked by 1 mM RuR. At this level, the Ca current would be completely blocked, so one would not expect transmission to be possible. These results lend support to the hypothesis that RuR blocks transmitter release from synaptosomes (Tapia and Meza-Ruiz, 1977) and the neuromuscular junction (Alnes and Rahamimoff, 1977; Person and Kuhn, 1979) by blocking Ca\(^{2+}\) entry.

The findings of Baux et al. (1978, 1979) are difficult to interpret considering the results presented here. While looking at Ca spikes in Aplysia neurons, they found that even 20 mM RuR does not block Ca-dependent action potentials. It could be that species differences in the Ca channels are responsible for this discrepancy. Another possible explanation is that the higher ionic strength of the artificial seawater, especially the higher divalent cation concentration, interferes with the blocking action of RuR on the Ca current. Person and Kuhn (1979) showed that Ca\(^{2+}\) and RuR do competitively interact. Thus, the higher Ca\(^{2+}\) and Mg\(^{2+}\) concentrations might inhibit the blocking action of RuR.

RuR Action on Na-Channel Inactivation

RuR slows inactivation of the Na channel with a half-maximal effect at 2.9 nM. This effect is partially reversible and quite specific. At 10 nM RuR, the only observed change in membrane properties is the greatly reduced rate of inactivation of the Na channel. The action of RuR to slow Na-channel inactivation has not been reported elsewhere. This may be of interest to those studying the kinetics of the Na channel. RuR may be a better agent for slowing Na-channel inactivation because it acts quickly and at a very low concentration. Recently, Nonner et al. (1980) tested a wide range of chemical agents that affect inactivation. They found that Na-channel inactivation was slowed to various degrees with treatments of 10 min with external 0.7 mM N-bromoacetamide, 36 min with internal iodate, 38 min with 40 mM external formaldehyde, 43 min with 1.8 mM external glutaraldehyde, or 45 min with 28 mM external pH 5 acetate buffer. Clearly, all of these agents take much longer and require much greater concentrations to be effective than does RuR.

The inactivation gate of the Na channel is believed to be located at the inner surface of the membrane mainly because pronase irreversibly removes inactivation when applied internally but not externally (Armstrong et al.,
1973). Baux et al. (1978, 1979) showed that intracellular RuR acts in *Aplysia* neurons at concentrations two to three orders of magnitude lower than those effective extracellularly. This suggests that the site of action of RuR may be on the cytoplasmic side of the membrane. Internal application of RuR was not attempted for several reasons. (a) The action of RuR on both the Na and Ca channels was complete within 30 s of bath application. This would suggest an extracellular site of action because such a highly charged molecule as RuR would not be expected to pass rapidly through the membrane. (b) RuR is very difficult to remove from the apparatus. In early experiments, it was found that RuR contamination in the bath chamber and the extracellular perfusion tubing was enough to saturate the effect on Na-channel inactivation. Thus, intracellular application of RuR would necessitate the replacement of the suction electrode apparatus for each new cell. This would have been a costly and a time-consuming process. (c) Because our primary interest in RuR was in its action on the Ca channel and our results showed it to be a nonspecific Ca-channel blocker, we decided not to explore further its mechanism of action. However, we do believe that the action of RuR to slow Na current inactivation may be useful in the analysis of Na-channel inactivation in other preparations, such as the squid giant axon. The fast action and specific binding of RuR reported here make it an excellent candidate for studying Na-channel inactivation.

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