

Mechanism of Action of "Ruthenium Red" Compounds on Ca^{2+} Ionophore from Sarcoplasmic Reticulum ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-Adenosine Triphosphatase and Lipid Bilayer*

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SUMMARY

Sarcoplasmic reticulum ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase was previously shown to have Ca^{2+} -dependent and -selective ionophoric activity when tested in oxidized cholesterol lipid bilayer membranes (Shamoo, A. E., and MacLennan, D. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 3522). Ruthenium red, a known inhibitor of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase, is found to inhibit the Ca^{2+} -ionophoric activity associated with ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase. Furthermore, ruthenium red alone acts as an anion-selective ionophore in lipid bilayers with the following selectivity sequence for anions: $\text{I}^- > \text{Cl}^-$, $\text{Br}^- > \text{F}^- > \text{NO}_3^-$. The $P_{\text{Cl}^-}/P_{\text{Na}^+}$ ratio was $\sim 4/1$. The presence of ruthenium red in excess of Ca^{2+} ionophore in lipid bilayer experiments converts the cation selectivity of the bilayer due to Ca^{2+} ionophore into anion selectivity.

Ruthenium red is a powerful inhibitor of Ca^{2+} transport in mitochondria (1-5). It is also a specific inhibitor of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase of red blood cells (6), and of Ca^{2+} binding and ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase of sarcoplasmic reticulum of rabbit skeletal muscle (7-8).

Shamoo and MacLennan (9) have shown that a Ca^{2+} -dependent and -selective ionophore is associated with the sarcoplasmic reticulum ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase from rabbit skeletal muscle. In view of the known action of ruthenium red on ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase we tested its effect on the Ca^{2+} ionophore from ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase. We found that ruthenium red (as an impure compound) has two different effects: (a) it acts as a highly selective anion ionophore in lipid bilayer and converts a cation-selective bilayer (due to the presence of Ca^{2+} ionophore) into an anion-selective bilayer; (b) it inhibits the Ca^{2+} ionophore action on bilayers when the Ca^{2+} ionophore is pretreated with ruthenium red.

MATERIALS AND METHODS

Sarcoplasmic reticulum ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase from rabbit skeletal muscle was isolated as described (10). Succinylation of the whole enzyme greatly increased its aqueous solubility, although it has been shown that the Ca^{2+} -dependent and -selective ionophoric activity is not a result of succinylation (9). All of the experiments were performed with succinylated enzyme.

The lipid bilayer was formed from oxidized cholesterol (11). The

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electronic circuitry consisted of a high impedance electrometer (Keithley model 616) in series with the black lipid membrane and a voltage source. We continuously measured conductance, capacitance, and cation:anion selectivity by imposing a salt gradient and a voltage clamp with triangular waveform of ± 70 mv at 0.015 Hz. The selectivity was estimated from the voltage intercept of the current-voltage relationship. For further details of the method, see previous references (9, 12).

Commercial preparations of ruthenium red may contain less than 20% of the pure compound (13). Furthermore, some companies label as ruthenium red a compound which is not defined as such by the original literature (13-15). We have identified ruthenium red, $[(\text{NH}_3)_5\text{Ru}-\text{O}-\text{Ru}(\text{NH}_3)_4-\text{O}-\text{Ru}(\text{NH}_3)_5]\text{Cl}_6$, molecular weight 786.130, according to the original literature (13-15). We obtained four commercial samples: two samples from Sigma Chemical Co. (St. Louis, Mo.) and one sample from each of the following: Ventron-Alfa (Beverly, Mass.), and ICN-K&K Pharmaceuticals (Plainview, N. Y.). The samples from Ventron-Alfa and ICN-K&K were not ruthenium red but tetraaminorutheniumhydroxylchlorochloride $[\text{Ru}(\text{NH}_3)_4\text{OHCl}]\cdot\text{Cl}\cdot 2\text{H}_2\text{O}$, molecular weight 293.14.

RESULTS AND DISCUSSION

Fig. 1 presents the data on bilayer conductance versus time in the presence of succinylated ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase as the Ca^{2+} ionophore. The bilayer was bathed with 50 mM CaCl_2 on one side and 5 mM CaCl_2 on the other side. A continuous current-voltage relationship was recorded due to a triangular voltage (0.015 Hz) imposed on the membrane. A complete one-way sweep of the current-voltage relationship was obtained in about $1/2$ min. The conductance change in that period was negligible. However, even if the conductance was increasing during the sweep, no change in the intercept was observed. Thus, the selectivity ratio was independent of conductance once the conductance was higher than the leakage conductance. Before the addition of ruthenium red the $P_{\text{Ca}^{2+}}/P_{\text{Cl}^-}$ was $\sim 4/1$ (see Curve A in Fig. 2). After the addition of ruthenium red (10^{-4} M) either the conductance continued to increase (not shown) or decreased slightly and then increased (as shown in Fig. 1). The selectivity changed from $P_{\text{Ca}^{2+}}/P_{\text{Cl}^-} \approx 4/1$ to

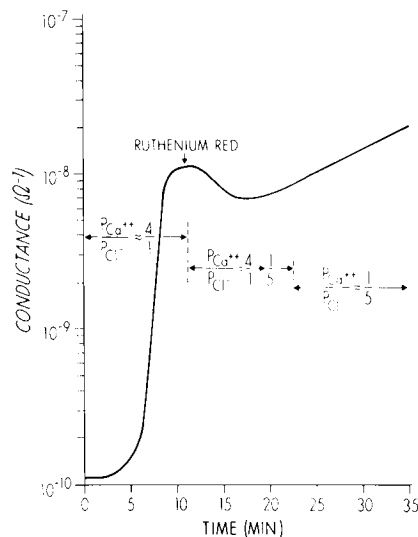


FIG. 1. Conductance of oxidized cholesterol bilayer membrane versus time in the presence of 2.6×10^{-3} mg/ml of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-ATPase ionophore on both sides of the membrane with 50 mM CaCl_2 on one side and 5 mM CaCl_2 on the other side. Histidine (5 mM), pH 7.3, was on both sides. At about 10 min, 10^{-4} M ruthenium red was added to both sides of the bilayer. The conductance and I-V curves were continuously monitored by imposing a triangular voltage clamp of ± 70 mv at a frequency of 0.015 Hz.

$P_{Ca^{2+}}/P_{Cl^-} \approx 1/5$ within 10 min and remained constant (see *Curve B* in Fig. 2). Thus, the lipid bilayer changes its properties from cationic selectivity to anionic selectivity. *Curve C* in Fig. 2 indicates that ruthenium red endows the lipid bilayer with anion selectivity in the absence of $(Ca^{2+} + Mg^{2+})$ -ATPase ionophore similar to that in the presence of the Ca^{2+} ionophore and ruthenium red. Next, we wanted to differentiate between the following modes of ruthenium red action: (a) ruthenium red might overwhelm the Ca^{2+} ionophore in the bilayer with anion-selective pathways, (b) ruthenium red might convert the cation-selective channel within the ionophore into an anion-selective channel, and (c) ruthenium red might inhibit the Ca^{2+} ionophore and also act as an anion ionophore.

We then proceeded to preincubate concentrated Ca^{2+} ionophore (2.6 mg/ml) with the same concentration of ruthenium red as in Fig. 1 (10^{-4} M). Aliquots of the pretreated Ca^{2+} ionophore were then added to the bathing fluid of the bilayer to give 2.6×10^{-3} mg/ml of Ca^{2+} ionophore. The final concentration of ruthenium red (10^{-7} M) in the bathing fluid due to the addition of the pretreated Ca^{2+} ionophore was less than ($1/1000$) that in Fig. 1. We have determined that at such low concentration ruthenium red has no direct effect on bilayer conductance. The result of this experiment was that there was no increase in lipid bilayer conductance. This indicates that ruthenium red completely blocks the action of Ca^{2+} ionophore. We increased the pretreated Ca^{2+} ionophore concentration to a level 5 times higher than that normally used in order to look for a strong effect on lipid bilayer conductance. The results even at such high dosage were negative. The aforementioned results indicate that ruthenium red has two different effects: (a) it inhibits the Ca^{2+} ionophore and (b) it acts as an anion-selective ionophore in lipid bilayer.

In order to measure the diffusion potential we started with equal concentrations of ions on both sides of the membrane followed by the addition of small volumes of highly concentrated salt to one side to create a salt gradient. It was observed that, after reaching a steady state conductance, a further increase in conductance occurred following the formation of the salt gradient (Fig. 3).

Table I gives average values of the permeability ratio of

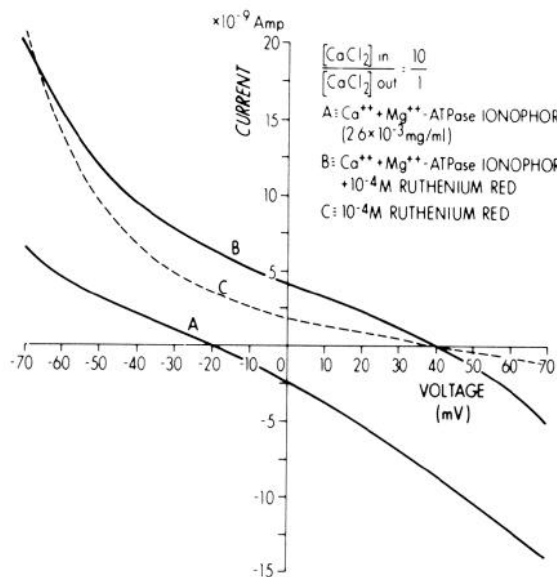


FIG. 2. Current-voltage relationship of oxidized cholesterol bilayer under three separate experimental conditions. In all three cases the bilayer was bathed with 50 mM $CaCl_2$ + 5 mM histidine (pH 7.3) inside, and 5 mM $CaCl_2$ + 5 mM histidine (pH 7.3) outside. In A both sides of the bilayer bathing fluid contained 2.6×10^{-3} mg/ml of $(Ca^{2+} + Mg^{2+})$ -ATPase ionophore in addition to Ca^{2+} . In B, the conditions were as in A plus 10^{-4} M ruthenium red. In C, 10^{-4} M ruthenium red was present, but there was no $(Ca^{2+} + Mg^{2+})$ -ATPase ionophore. The experimental measurements were made in a manner similar to that described for Fig. 1.

chloride to either Ca^{2+} or Na^{2+} when measured by the diffusion potential. The lower selectivity value for P_{Cl^-}/P_{Na^+} than for $P_{Cl^-}/P_{Ca^{2+}}$ may be due to leakage of Na^+ . To find the anion-selectivity ratio of the bilayer in the presence of ruthenium red we tested the bi-ionic potential in the presence of 5 mM $CaCl_2$ + 5 mM histidine (pH 7.3) versus 5 mM $Ca(NO_3)_2$ (or $CaBr_2$ or CaI_2) + 5 mM histidine (pH 7.3). In the case of fluoride we used 5 mM $NaCl$ versus 5 mM NaF (Table II).

In the case of $Ca(NO_3)_2$ versus $CaCl_2$ we found that the bilayer had to be formed from the $CaCl_2$ side in order to achieve an increase in conductance when ruthenium red was present on both sides of the membrane. The rate of increase in bilayer conductance due to ruthenium red under such conditions was lower than that when $CaCl_2$ and ruthenium red were present on both sides of the bilayer. As a matter of fact, if $Ca(NO_3)_2$, $CaCl_2$, and ruthenium red were present on both sides of the bilayer, no increase in conductance was observed. The presence of compounds like sulfate, nitrate, and oxalate on one or both sides of the bilayer at concentrations equal to $CaCl_2$ caused the inhibition of ruthenium red action on the bilayer when ruthenium red was present on both sides of the bilayer. ATP was also found to be inhibitory to the effect of ruthenium red on the bilayer. This may indicate that multivalent anions form complexes with ruthenium red (6+) preventing its incorporation into the bilayer or its complexation with monovalent anions like chloride. The ruthenium red in a multivalent anion complex may become immobile in the membrane. This may explain some of the inconsistent results ob-

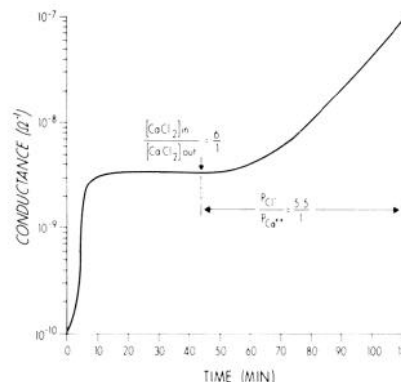


FIG. 3. Conductance of oxidized cholesterol bilayer membrane versus time in the presence of 10^{-4} M ruthenium red and 5 mM $CaCl_2$ + 5 mM histidine on both sides of the bilayer. At about 2 min a concentration gradient of $[CaCl_2]_{in}/[CaCl_2]_{out} = 6/1$ was created by adding concentrated $CaCl_2$ to the inside chamber. The experimental measurements were made in a manner similar to that described for Fig. 1.

TABLE I
Cation:anion selectivity

Cation:anion selectivity ratio of oxidized cholesterol black lipid membrane in the presence of 10^{-4} M ruthenium red + 5 mM histidine (pH 7.3) on both sides of the bilayer and the appropriate ions. In the first row we had 50 mM $CaCl_2$ inside versus 5 mM $CaCl_2$ outside. In the second row we had 50 mM $NaCl$ versus 5 mM $NaCl$. The experimental measurements were made in a manner similar to that described for Fig. 1.

$P_{Cl^-}/P_{Ca^{2+}}$	5.3 ± 0.17 $n = 6$
P_{Cl^-}/P_{Na^+}	3.9 ± 0.12 $n = 5$

TABLE II
Anion selectivity

Anion selectivity of black lipid membrane in the presence of ruthenium red (10^{-4} M) measured with respect to chloride ion, 5 mM of each anionic salt of calcium versus 5 mM $CaCl_2$ + 5 mM histidine (pH 7.3) on both sides. In the case of fluoride ion we used 5 mM NaF versus 5 mM $NaCl$. The experimental measurements were made in a manner similar to that described for Fig. 1.

Selectivity sequence	$I^- > Cl^- > Br^- > F^- > NO_3^-$
Selectivity ratio	$14.3 > 1.0 > 1.0 > 0.56 > 0.1$

tained with ruthenium red under different experimental conditions (1-7).

We tested two batches from Sigma and found that both batches gave similar results to that reported here for one of them. However, the results obtained from the batch not reported here required 10^{-3} M instead of 10^{-4} M ruthenium red in order to obtain the same lipid bilayer conductance increase obtained with the first batch. Tetraaminorutheniumhydroxychlorochloride from Ventron-Alfa and ICN-K&K at 10^{-2} M had no effect on the bilayer, nor did the pretreatment of the Ca^{2+} ionophore with these compounds have any effect on the conductance increase or cationic selectivity associated with the Ca^{2+} ionophore. Thus, our data indicate that the crude preparation of ruthenium red from Sigma has the properties of an anion-selective ionophore in lipid bilayers and an inhibitor of Ca^{2+} ionophore. This is consistent with the conclusions of Reed and Bygrave that purified ruthenium red (3) inhibited Ca^{2+} transport in mitochondria, and a low molecular weight contaminant of ruthenium red (16) inhibited Ca^{2+} transport in rat liver mitochondria.

This work demonstrates that the crude ruthenium red, as commercially available, endows the lipid bilayer with highly specific anionic selectivity and inhibits Ca^{2+} ionophoric activity. The two different actions may be due to two different compounds in crude ruthenium red.

This work also suggests that a possible mechanism of ruthenium red action in inhibiting $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase of sarco-

plasmic reticulum or inhibiting Ca^{2+} transport in mitochondria is the inhibition of the Ca^{2+} carrier. We do not know whether ruthenium red causes biological membranes to become anion-selective.

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