Inhibitory Properties of Ruthenium Amine Complexes on Mitochondrial Calcium Uptake[1]

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The recent finding that the inhibition of Ca^{2+}-stimulated respiration by ruthenium red is mainly due to a binuclear ruthenium complex (Ru_{360}) present in the commercial samples of the classical inhibitor ruthenium red (Ying *et. al.*, 1991), showed that this complex is the more potent and specific inhibitor of the mitochondrial calcium uniporter. This work was aimed to provide insights into the mechanism by which Ru_{360} and other ruthenium-related compounds inhibits calcium uptake. Ruthenium red and a synthesized analog (Rrphen) were compared with Ru_{360}. The inhibition by this binuclear complex was noncompetitive, with a K_i of 9.89 nM. The number of specific binding sites for Ru_{360} was 6.2 pmol/mg protein. Ruthenium red and Ru_{360} were mutually exclusive inhibitors. Bound La^{3+} was not displaced by Ru_{360}. Rrphen was the least effective for inhibiting calcium uptake. The results support the notion of a specific binding site in the uniporter for the polycationic complexes and a negative charged region from the phospholipids in the membrane, closely associated with the uniporter inhibitor-binding site.

KEY WORDS: Calcium uniporter inhibitors; mitochondria.

INTRODUCTION

Cyclic accumulation and release of calcium occur through the action of the Ca^{2+} uniporter and the opposing transporters; thus, the $[Ca^{2+}]_m$ is strictly regulated. The matrix calcium concentration is thought to participate in the control of NADH production by the TCA cycle and, hence, in the regulation of the ATP synthesis by mitochondria (McCormack *et al.*, 1990; Hansford, 1994). It is claimed that matrix calcium concentration also exerts control in the rate of ATP synthesis by acting on the electron transport (McCormack *et al.*, 1990; Panov and Scaduto, 1995), on the adenine nucleotide translocase (Moreno-Sánchez, 1985; Halestrap,

1987), and over the ATPase per se (Yamada and Huzel, 1988; Panov and Scaduto, 1995).

In recent studies, there has been growing evidence that the mitochondrial calcium cycle participates in regulation of cellular Ca^{2+} levels by signaling mechanisms, under physiological conditions. It has been shown that engineered cell lines that express aequorin in mitochondria accumulate calcium in these organelles from an intracellular domain of high calcium concentration (Rizzuto *et al.*, 1993). Other reports indicate that in neurons, mitochondrial Ca^{2+} transport is quantitatively important in regulating the cytoplasmic as well as the matrix Ca^{2+} concentration (White and Reynolds, 1995). Recognition that the mitochondrial calcium cycle plays an important role in physiological processes has renewed interest in the study of the physical properties of the transporters involved.

Much of the information cited above is based on the specificity of inhibitors of mitochondrial calcium uptake, e.g., ruthenium red. Ying *et al.* (1991) purified a compound present in commercial samples of ruthenium red, which was identified as an oxo-bridged binuclear amino complex, which inhibited Ca^{2+}- stimulated respira-

tion with higher potency than ruthenium red itself. Besides the recent report of Matlib *et al.* (1998), the effect of Ru_{360} on calcium uptake in mitochondria has not been yet determined. The present work was conducted to determine a possible mechanism of action of the polycationic inhibitors of mitochondrial calcium uptake by comparing the effectiveness and sites of action of Ru_{360} with ruthenium red. An analog with bulky phenanthroline substituents (Rrphen) was synthesized and its potency assayed to define the requirements of a basic structure for the calcium uniporter inhibitors.

EXPERIMENTAL.

The synthesis of Ru_{360} was as described by Ying *et al.* (1991), by the reaction of $RuCl_3$ with ammonium hydroxide; the separation of the formed complexes was achieved by cation-exchange chromatography. The purified preparation was slightly yellowish and exhibited a single λ_{max} at 360 nm in ammonium formate. The radioactive complex $^{103}Ru_{360}$ was obtained from $^{103}RuCl_3$ by the same method. Commercial ruthenium red was purified by the technique described by Luft (1971). This preparation was not contaminated with Ru_{360}. A single absorption peak at 533 nm was observed with distilled water.

The related compound with phenanthroline substituents was synthesized by using ruthenium red, 1,10-phenanthroline monohydrate and ammonium hexafluorophosphate.

Rrphen

Ruthenium red (0.6032g) was dissolved in distilled water (15 ml) and the solution filtered; the solid material was discarded. To this a solution, 4.5 g of 1,10-phenanthroline monohydrate in methanol (30 ml) was added. This solution was stirred for 24 h under nitrogen; the solution was kept at 45°C for another 6 h. When the solution cooled down, the solvent was evaporated into a high vaccum line until the remaining volume was about 15 ml. To this, an aqueous solution of 0.75 g of hexafluorophosphate ammonium salt was added in order to precipitate the complex. The solid formed was filtered under nitrogen and washed with abundant ethanol until the washings were colorless. The solid was further washed with ethanol (500 ml) in order to ensure the removal of any remaining free phenanthroline.

The product was allowed to dry under vaccum and nitrogen atmosphere. Elemental analysis found: C, 48.41; N, 13.64; and H 3.61%; $C_{96}Cl_4F_{12}H_{82}N_{22}O_2$-$P_2Ru_3$ requires C, 49.9; N, 13.34, and H, 3.58%.

Infrared spectra were recorded from KBr pellets of the complex with a Perkin Elmer 599 B spectrophotometer in the range 4000–450 cm^{-1}. Electronic spectra were measured with a Hewlett Packard 8452 Diode Array spectrophotometer. [^{1}H], [^{13}C], [^{31}P], and [^{19}F] NMR spectrometry were obtained in dimethyl-d_6 sulfoxide solutions using a Varian NMR Unity plus 500 spectrometer.

Mitochondria from rat kidney were prepared by differential centrifugation as described by Chávez *et al.* (1985). The last wash was carried out in a medium containing 0.1% bovine serum albumin in 250 mM sucrose and 10 mM Tris, pH 7.3. The final pellet was suspended in the same medium without albumin. Protein concentration was determined by the Biuret method using bovine serum albumin as a standard. The samples were solubilized with deoxycholate, which was present at a final concentration of 1% by weight.

Calcium uptake was measured using $^{45}CaCl_2$ (specific activity, 1000 cpm/nmol) following the filtration technique through Millipore filters of 0.45-μm pore diameter, at the indicated time. The assay medium contained 125 mM KCl, 10 mM HEPES, 10 mM succinate, 200 μM ADP, 10 μg rotenone, 2 mM Pi, and 50 μM $^{45}CaCl_2$, pH 7.3. Ru_{360} or the related compounds at the desired concentration, were added before Ca^{2+} uptake was initiated by the addition of 1 mg of mitochondrial protein. Binding experiments were carried out with isolated mitochondria (1 mg) incubated at 25°C in 6 ml of medium containing 125 mM KCl, 10 mM succinate, and 10 mM HEPES, pH 7.3, and 2–30 nM $^{103}Ru_{360}$. After 10 min of incubation, the samples were centrifuged at 20,000 × g for 10 min and the supernatant discarded. The pellet was resuspended in the same buffer and centrifuged again at 20,000 × g for 10 min. The pellet was resuspended in the wash medium with 10 μM of unlabeled Ru_{360}, and incubated for 1 min to eliminate nonspecific binding of the inhibitor. After a final centrifugation, the pellet was resuspended in a small volume of medium, transferred into a test tube, and its radioactivity content measured in a gamma counter.

RESULTS AND DISCUSSION

The structures of ruthenium red, Ru_{360}, and the synthesized analog are shown in Fig. 1. A 16 μM
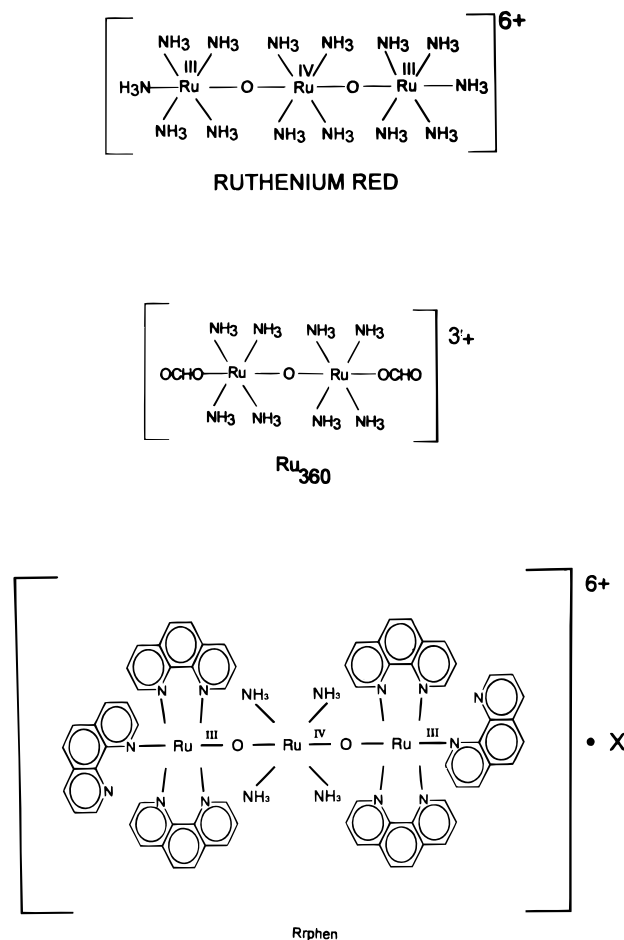
Fig. 1. The structures of ruthenium red and Ru_{360} (from Carrondo *et al.*, 1980 and Ying *et al.*, 1990, respectively). A possible arrangement of the phenanthrolines in the compound Rrphen is also shown.

solution of Ru_{360} exhibits an absorbance maximum at 360 nm, with no detectable absorbance at 533 nm, indicating that there is no contamination with ruthenium red. The spectrum of purified ruthenium red is also free of absorbance at 360 nm. Both compounds were free of cross contamination and were used to inhibit energized calcium uptake. The purified ruthenium red complex inhibited with a K_i similar to that reported by Reed and Bygrave (1974) (30 nM; data not shown).

The [¹H] NMR spectra at 297 K of Rrphen shows the same pattern found for the free phenanthroline. However, in our complex, the peaks are shifted downfield, indicating that the phenanthroline ligands are coordinated. Since we liked to confirm that the hexafluorophosphate counterion had not suffered any redox reaction, a ³¹P and a ¹⁹F resonance spectra were

recorded. The results confirm that the PF_6 counterion remains unchanged. The visible spectrum for Rrphen shows a main peak at 534 nm, assigned to the charge transfer electronic transitions. In the UV region, strong signals appear, which correspond to phenanthroline. The infrared spectrum of KBr pellets of the Rrphen complex in the range 4000–450 cm−1 shows bands at 3300–3100, 1620, and 1296, which correspond to the N–H group. The bands of the phenanthroline appears slightly shifted, confirming their coordination (not shown).

The potency of the binuclear complex Ru_{360} was assayed in energized mitochondria, under the conditions described. The uniporter had a very high affinity for the inhibitor; the K_i obtained was 7.68 nM, as we previously reported (Zazueta *et al.*, 1998). The inhibitor was considered tightly bound. Therefore, the analysis was made by the Dixon graphical method (Segel, 1993) for this type of inhibitors (Fig. 2). The K_i obtained was 9.89 nM and the concentration of the inhibitor that is bound to the uniporter was calculated
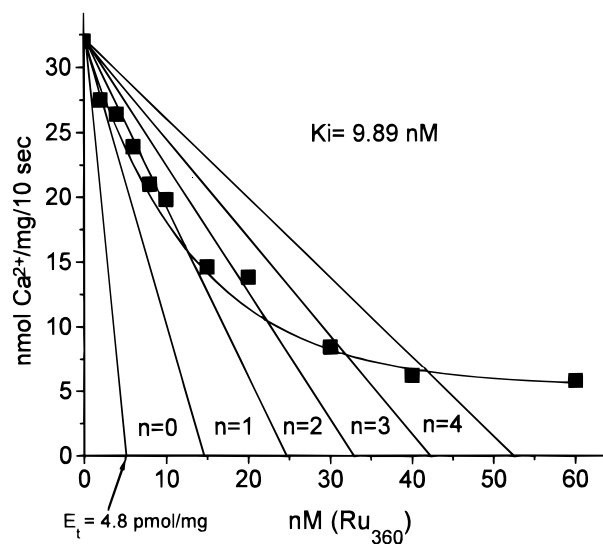

Fig. 2. Dixon graphical plot for tightly bound inhibitors. Calcium uptake was inhibited in the presence of different concentrations of Ru_{360} under the conditions described in the experimental section (■). A set of lines were drawn on the experimental data from v_o to the $[I]_t$ axis, in such a way that each line intersects the experimental curve at $v_{o/2}$, $v_{o/3}$, $v_{o/4}$, and so on. This represent a 1/2, 1/3, and 1/4 of the activity. The distance between the intercepts on the $[I]$ axis gives K_i directly. The line drawn from v_o to the $[I]$ axis two $K_{i's}$ distance from the left of 1/2 v_o, represents the $[I]_o$ of the total concentration inhibitor. Finally the distance between this line and the y axis is the $[E]_t$ where a significant proportion of the inhibitor is bound. $[I]_t$ = Total free inhibitor concentration. $[E]_t$ = Total enzyme concentration.

as 4.8 pmol/mg protein. These results are slightly different from those reported recently by Matlib *et al.* (1998) for a compound identified as Ru_{360}. They reported an $IC_{50\%}$ of 0.184 nM and a maximum binding of 80 fmol/mg of protein with a dissociation constant of 0.34 nM in isolated rat heart mitochondria, measuring calcium movements spectrophotometrically with arsenazo III. Indeed, the compound analyzed in this work exhibited more potency than pure ruthenium red.

The binding of $^{103}Ru_{360}$ was also determined by adding the inhibitor directly to mitochondria in 125 mM KCl, 10 mM TRIS, pH 7.3, as described in the experimental section. The binding curve was hyperbolic and appeared to be a saturable function above 20 nM (Fig. 3a). Specific binding determined by a Scatchard plot gave a value for B_{max} of 6.2 pmol/mg mitochondrial protein; the K_d was 16.5 nM (Fig. 3 insert). The relation between binding of the binuclear ruthenium ammonium complex and inhibition of calcium uptake showed a clear correspondence.

The large variety of ruthenium amino compounds, obtained by ammoniacal reaction mixture from which ruthenium red is synthesized, raised the question as to whether all complexes are inhibitors of the calcium uniporter. Although they are structurally related, the inhibitory potency between the hexacationic ruthenium red and the lesser cationic charged Ru_{360} is almost 25-fold. The potencies of the remaining complexes have been already analyzed (Reed and Bygrave, 1974). The experiment shown in Fig. 4, was conducted to define the competitive behavior between ruthenium red and
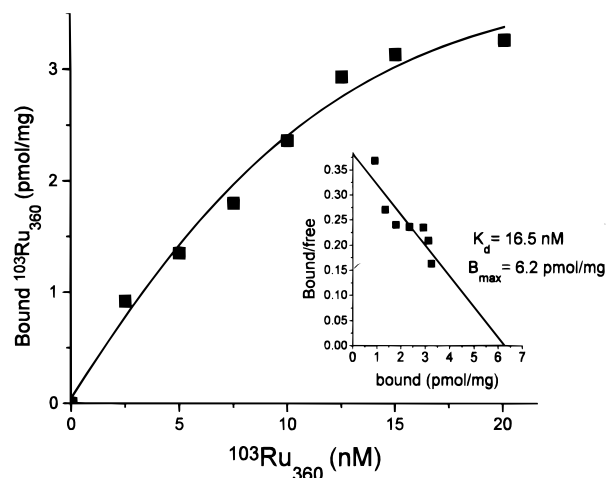

Fig. 4. Inhibition of mitochondrial calcium uptake by Ru_{360} in the presence of ruthenium red. (a) Inhibition of Ru_{360} in the presence of increasing concentrations of ruthenium red at a fixed Ca^{2+} concentration. (b) Dixon plot for the inhibition values (■, 0 ruthenium red; ●, 50 μM ruthenium red; ▲, 75 μM ruthenium red; ♦, 100 μM ruthenium red; and ▼, 150 μM ruthenium red. (c) Dixon graphic replot of the data without Ru_{360} renders the K_i for ruthenium red. Calcium uptake determinations were made by using $^{45}CaCl_2$, as described.

Ru_{360} for the uniporter binding sites. The inhibition by Ru_{360} in presence of different fixed concentrations of ruthenium red is shown in Fig. 4a. The mutual exclusivity of Ru_{360} and ruthenium red is evident from the Dixon plots of $1/V$ versus the concentration of the binuclear complex at a fixed 50 μM calcium concentration and different fixed concentrations of ruthenium red (Fig. 4b). The slopes of the plots are independent of the concentration of the fixed (ruthenium red) inhibitor. The family of curves are parallel and the intercepts on the $1/V$ axis represent the inhibition of ruthenium red at different concentrations. The replot of these values against the fixed ruthenium red concentrations, represents the usual Dixon plot for this inhibitor. The K_i obtained was 25 nM (Fig. 4c). The pure noncompetitive

Fig. 3. Equilibrium binding of $^{103}Ru_{360}$ in rat kidney mitochondria. The insert shows the Scatchard plot of the specific binding of $^{103}Ru_{360}$. The conditions were as described in the experimental section.
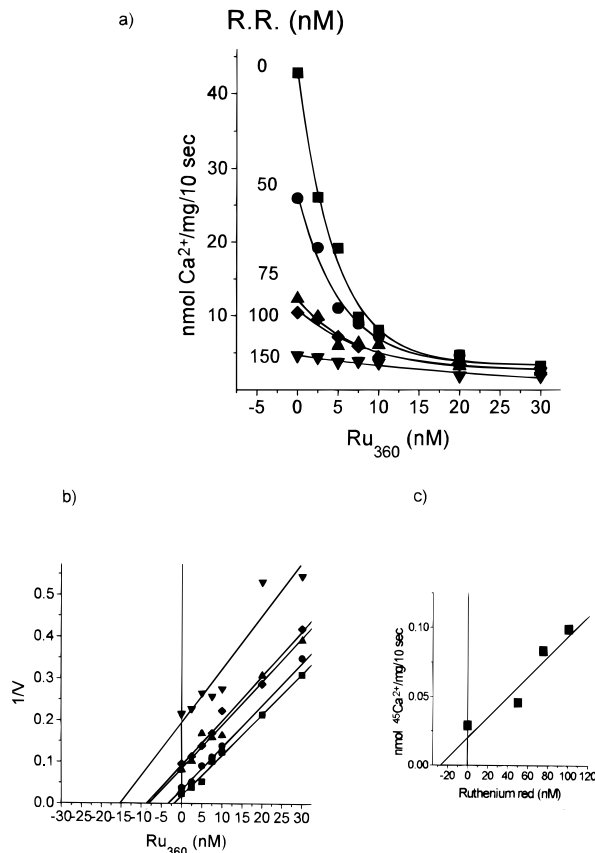
character of both inhibitors is demonstrated in this figure: Dixon plots from partial and most mixed non-competitive inhibitors are curved. When the complex enzyme–substrate inhibitor is not catalytically active, the plot is linear. The addition of Ru_{360} to the system did not promote a synergistic effect on the inhibition. The same occured when the fixed inhibitor was Ru_{360} versus different concentrations of ruthenium red (not shown).

It has been reported that ruthenium red binds to the so-called "low-affinity" calcium-binding sites that lanthanum (a competitive inhibitor of the calcium uniporter) can also occupy. These sites are presumably phospholipids. Voelker and Smetjck (1996) demonstrated that ruthenium red behaves in a diffuse double layer as an ion with an effective valence < 6 and has a very high affinity for phosphatidylserine-containing membranes. Does Ru_{360} compete with lanthanum for this low-affinity calcium-binding site?

A competition assay was performed to determine if Ru_{360} behaves as ruthenium red, in the presence of lanthanum. The addition of lanthanum to a fixed concentration of Ru_{360} promoted a larger inhibition than of the polynuclear complex by itself (Fig. 5a). The Dixon plot (Fig. 5b) shows that the high-affinity binding sites for La^{3+}, with a K_i of 100 nM were not modified when the oxo-bridged binuclear complex was present in the incubation medium; as expected only the low-affinity binding sites were modified in presence of Ru_{360}. The conclusion that could be drawn is that Ru_{360} displaces La^{3+} from the nonspecific phospholipid sites, but not from the calcium carrier binding sites.

The amine complexes of trivalent cobalt are similar in many respects to ruthenium complexes. Tash-

mukhamedov *et al.* (1972) have reported that hexamine cobalt chloride $[Co(NH_3)_6]^{3+}$ inhibits mitochondrial calcium transport. This complex cation was reported to react with the negatively charged sites of sulfate-containing glycosaminoglycans and is used for determining the total amount of their anionic groups. The half-maximum inhibition observed was 3×10^{-5} M and this represents one order of magnitude higher than the inhibition exerted by ruthenium red.

In an aqueous environment, ruthenium red is present as an hexavalent cation (Carrondo *et al.* 1980), which due to its highly positive charge, is expected to be very responsive to negative charges on proteins, lipid membranes, and cation carriers. The possibility of electrostatically mediated action of ruthenium red in biomembranes in relation to its adsorption to negatively charged lipids has already been discussed in the literature. Missiaen *et al.* (1990) proposed that the inhibition of the Ca^{2+} pump in the plasma membrane by ruthenium red is due to an electrostatic origin mediated by the neutralization of negatively charged phospholipids by adsorbed ruthenium red cations.

To define the requirement of a basic structure for the calcium uniport inhibitors and to discard a pure electrostatic mechanism, the potency of an analog of the polynuclear complexes was assayed. The compound possesses the essentially linear backbone N-Ru-O-Ru-O-Ru-N formed from three coordinated ruthenium, however, some of the amino groups are substituted by phenanthrolines groups. The net charge of the compound is $+6$. It inhibits calcium uptake with a K_i of approximately 0.3 μM (not shown). This represents about tenfold the K_i reported for ruthenium red and thirty-fivefold the K_i obtained for Ru_{360}. This complex forms a very voluminous compound that presumably cannot fit adequately in the conformed space that is occupied by the almost cylindrical ruthenium red molecule (Carrondo *et al.*, 1980). Ru_{360} has the same linear backbone N-Ru-O-Ru-N and each ruthenium atom is positively charged with the remaining charge delocalized between the Ru-O-Ru bridge (Matlib *et al.*, 1998). A possible model in Fig. 6, is proposed to explain the inhibitory properties of ruthenium amino complexes. The uniporter molecule has been considered to be a fast "gated" pore (Sparagna *et al.*, 1995; Litsky and Pfeiffer, 1997) with a recognition binding site for calcium, located near the cytoplasmic side of the membrane (Fig. 6a). The inhibitors described did not compete with calcium for the high-affinity sites related to the transporter, but they did so to the low-affinity sites for calcium, represented by acidic phospholipids.
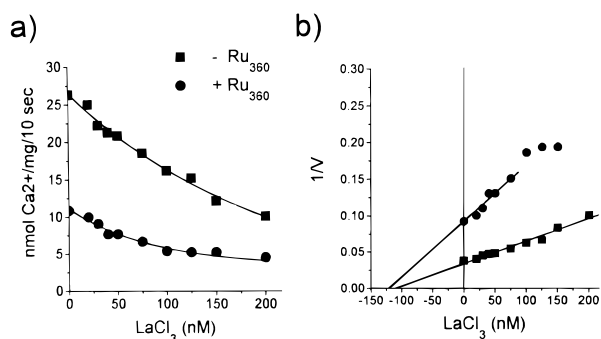

Fig. 5. Inhibition of calcium uptake by Ru_{360} in the presence of lanthanum. Calcium movement was determined as described in the experimental section. The medium contained 125 mM KCl, 10 mM HEPES, 10 mM succinate, 200 μM ADP, 10 μg rotenone, 40 nM Ru_{360}, and 50 μM $^{45}CaCl_2$. Phosphate was not added to the medium. ■, Without Ru_{360}; ●, plus 40 nM Ru_{360}.
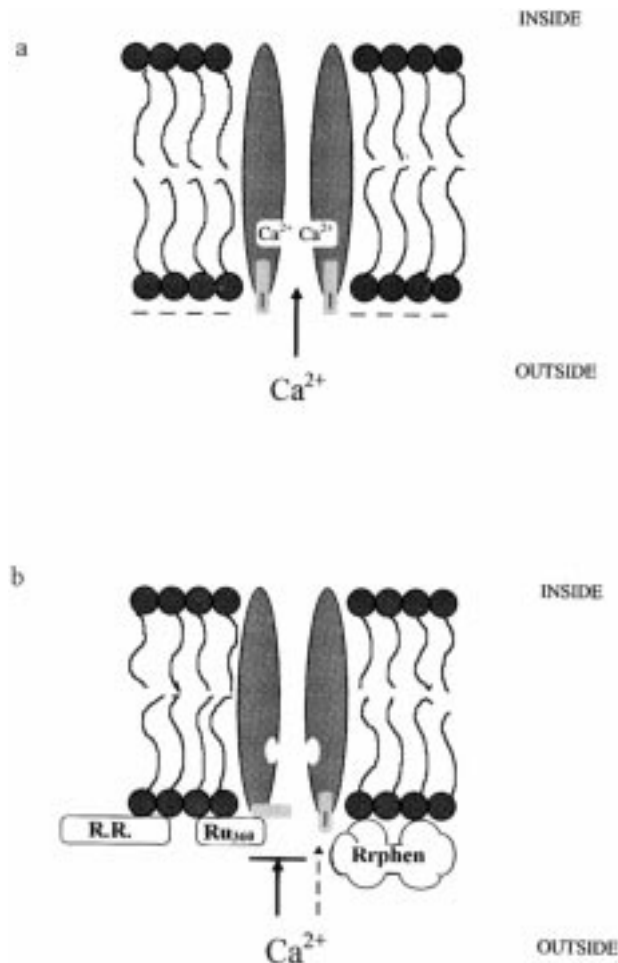
Fig. 6. Proposed model to explain the inhibitory properties of the polycationic ruthenium complexes described. (a) The uniporter molecule embedded in the membrane with two sites for calcium binding and a negatively charged density region, facing the outside of the inner membrane. (b) The polycationic complexes present different ability to fit this negatively charged density region. The 0.8 nm molecule of Ru_{360} binds to this site and exerts a conformational change that modifies the recognition site for calcium; this compound also binds to the phospholipids, so it could be anchored to the membrane as well as to the uniporter-binding site. The 1.2 nm length ruthenium red possesses a higher affinity for acidic phospholipids, because of its hexacationic charge, but it could, indeed, fit in the uniporter-binding site. Finally, the bulky phenanthroline analog exerts low inhibition on calcium uptake, perhaps for its inability to promote a direct conformational change on the uniporter, but instead modifies the low-affinity binding sites for calcium at the phospholipid level.

The uniporter is a cooperative mechanism in which calcium binding to an activation site increases the rate of transport. The high specificity for ruthenium red and Ru_{360} suggests that the uniporter cytoplasmic side possesses a highly negatively charged density region,

where the cylindrical complexes bind and promote a conformational change that modifies the calcium activation site in such a way that the uptake would not be activated. The fact that both compounds are mutually exclusive and that Ru_{360}, as well as ruthenium red, displace lanthanum from the low Ca^{2+}-binding sites, supports the notion of the presence of a specific binding site in the uniporter and negative charges from phospholipids in the membrane. Ruthenium red can be regarded as a charged cylinder 1.2 nm long (Carrondo *et al.*, 1980) or as a linear array of three charges separated by 0.6 nm. The bridging Ru-O bond lengths and the Ru-N distances estimated on the basis of the X-ray structure of Ru_{360} Ying (*et al.*, 1991) reveal a cationic complex of approximately 0.8 nm of a cylindrical shape. In Fig. 6b, it is proposed that both complexes could be anchored to negative phospholipids closely associated with the uniporter inhibitor-binding site, in such a way that an adequate fitting to the carrier modifies the structure of the calcium channel. The lower affinity for ruthenium red could be explained in terms of its more positive charge that makes the complex very responsive to unspecific negative charges on proteins and lipid membranes. As the concentration of ruthenium red increased, unspecific screening to negative charges of the membrane diminished. The behavior of the ruthenium analog is explained on the basis of its bulky and more hydrophobic structure. Adsorption of ions to lipid membranes has been reviewed recently by Tatulian (1994). The low inhibitory potency of this complex could be related to their adsorption to the lipidic environment, as well to their steric inability to modify the Ca^{2+}-activation binding site.

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