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EFFECTS OF RUTHENIUM RED ON Ca^{2+} UPTAKE AND ATPase OF SARCOPLASMIC RETICULUM OF RABBIT SKELETAL MUSCLE

MARIA G. P. VALE and ARSÉLIO P. CARVALHO

Departamento de Zoologia, Universidade de Coimbra, Coimbra (Portugal) and Institute for Muscle Disease, New York, 10021, N.Y. (U.S.A.)

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SUMMARY

Ruthenium red, a powerful inhibitor of Ca^{2+} transport by mitochondria, does not inhibit the active Ca^{2+} uptake by sarcoplasmic reticulum isolated from rabbit skeletal muscle promoted by 5 mM ATP–Mg in the presence or absence of potassium oxalate. Although concentrations of ruthenium red up to 100 μM do not affect the active uptake of Ca^{2+}, 25 μM of the inorganic dye inhibit the passive binding of Ca^{2+} by about 50%. This inhibitory effect is observed in sarcoplasmic reticulum even after its lipid fraction is extracted with acetone.

Although active Ca^{2+} uptake by sarcoplasmic reticulum is not inhibited by ruthenium red, in the absence of oxalate it inhibits significantly the Ca^{2+}-dependent ATPase activity but not the Mg^{2+}-ATPase. However, if potassium oxalate is present, the Ca^{2+}-stimulated ATPase is not sensitive to the dye. It is not clear how oxalate functions to protect the Ca^{2+}-ATPase against the inhibitor effect of ruthenium red.

The high sensitivity to ruthenium red of the Ca^{2+} transport mechanism in mitochondria as compared to the Ca^{2+} transport in sarcoplasmic reticulum may be useful in determining the extent to which each organelle functions in the cell to regulate intracellular free Ca^{2+}.

INTRODUCTION

Sarcoplasmic reticulum regulates the Ca^{2+} distribution inside the muscle cell which permits the regulation of contraction and relaxation[1].

In the presence of ATP, isolated sarcoplasmic reticulum accumulates Ca^{2+} from the medium by a process dependent on the $(Mg^{2+}+Ca^{2+})$-ATPase activity[2–6], which reflects the coupling of ATP hydrolysis to Ca^{2+} movement[4,7]. It was originally assumed that the accumulated Ca^{2+} remains inside the vesicles as free Ca^{2+} (refs 1, 3), but results obtained in our labotarory[8] indicate that at least 80% of the Ca^{2+} accumulated is bound to the membranes, although it is not clear whether the Ca^{2+} is first transported and then bound to the internal side of the membranes, or the ATP induces selective binding of Ca^{2+} by the membranes. The passive Ca^{2+} binding capacity of the sarcoplasmic reticulum membranes indicates that they have enough binding sites to account for all the Ca^{2+} binding in the presence of ATP[8]. About

50% of the binding sites of the membranes are accounted for by the presence of phospholipids[9].

Recently, several works have been published about the powerful inhibitory effect of ruthenium red on the Ca^{2+} translocation in mitochondria[10–14] and work with skinned muscle fibers has suggested that this substance does not affect the Ca^{2+} uptake by sarcoplasmic reticulum[12]. Ruthenium red is an inorganic dye used as a histochemical stain which has been suggested to react selectively with mucopolysaccharides to form a stable red complex[15]. This dye, besides inhibiting the Ca^{2+} uptake supported by mitochondrial respiration or by ATP[10,11], also inhibits the high and low affinity passive binding of Ca^{2+} by mitochondrial membranes[11].

Recently, Gillis[12] showed that ruthenium red slows down the speed of relaxation of red muscle fibers and attributed the effect to the inhibition of Ca^{2+} transport by mitochondria, whereas ruthenium red does not affect those muscles in which the regulation of cytoplasmic free Ca^{2+} has been considered to depend exclusively on the activity of their sarcoplasmic reticulum. Therefore, it is of interest to determine whether ruthenium red does not, in fact, interfere with the Ca^{2+} accumulation of isolated sarcoplasmic reticulum. In this work we studied the effect of ruthenium red on the Ca^{2+} binding, Ca^{2+} uptake and ATPase of sarcoplasmic reticulum isolated from rabbit skeletal muscle.

METHODS

Isolation of sarcoplasmic reticulum

Sarcoplasmic reticulum vesicles were prepared from rabbit leg and back skeletal muscles by a modification of the method described previously[8].

Muscle mince was firstly centrifuged at a low speed ($8000 \times g$) and then the resultant supernatant containing sarcoplasmic reticulum was submitted at a high speed centrifugation ($40000 \times g$) for 1 h. The collected sarcoplasmic reticulum was washed once with the isolation medium (0.1 M KCl–5 mM imidazole at pH value of 7.0) and finally it was resuspended in 0.25 M sucrose–10 mM Tris at a pH value of 7.0 or in the isolation medium, and stored at 0 °C.

Lipid-free sarcoplasmic reticulum was obtained by extracting the phospholipids from the membranes by the method described by Fleischer and Fleischer[17].

Binding of Ca^{2+} by isolated sarcoplasmic reticulum

The passive binding of Ca^{2+} (in the absence of ATP) by isolated sarcoplasmic reticulum was carried out at room temperature (25 °C) in a standard medium containing 2.5 mg of protein, 10 mM imidazole at a pH value of 7.0 and 3 mM $CaCl_2$ in a final volume of 10 ml. After an equilibration period of 5 min, the reaction was stopped by centrifuging at $100000 \times g$ for 30 min at 0 °C. The pellets were rinsed three times with deionized water and were resuspended in 4 ml of water. This resuspension was used for analysis of Ca^{2+} and protein.

For the active Ca^{2+} uptake (in the presence of ATP) by sarcoplasmic reticulum, the suspension was incubated 10 min at room temperature (25 °C) in a standard medium containing 2 mg of protein, 10 mM imidazole at a pH value of 7.0, 5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ in a final volume of 10 ml. The reaction was started by the addition of ATP to a final concentration of 5 mM and stopped by centrifu-

gation at 100000 × *g* for 30 min. The pellets were then rinsed with deionized water and resuspended in 4.0 ml of water for Ca^{2+} and protein analysis.

Binding of ruthenium red by sarcoplasmic reticulum membranes

The capacity of sarcoplasmic reticulum membranes to bind ruthenium red was determined from the difference between the absorbance (measured at 540 nm) of the reaction medium before adding the suspension of sarcoplasmic reticulum (2 mg of protein), and the absorbance of the supernatant obtained after centrifuging the reaction mixture for 30 min at 100000 × *g*. A previous determination of a standard curve of ruthenium red showed that within the range of concentrations of ruthenium red used, there is a rectilinear relationship between concentration and absorbance. The reaction medium contained 10, 20, 30, 50 and 100 μM of ruthenium red in a final volume of 5 ml. When ATP was present, its concentration was 5 mM. The equilibration period was 5 min at room temperature (25 °C).

ATPase assay

ATPase activity was assayed by measuring the inorganic phosphate release from ATP during 10 min of incubation at room temperature (25 °C) in a standard medium containing 2.5 mg of protein, 10 mM imidazole at a pH value of 7.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA and 5 mM ATP in a final volume of 5 ml. In some cases, 5 mM of potassium oxalate was also present. The reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid. The Ca^{2+}- dependent ATPase is calculated by subtracting the Mg^{2+}-dependent activity (basic ATPase) from the activity measured in the presence of Mg^{2+} and Ca^{2+}. Phosphate was measured by the method described by Taussky and Shorr[18].

Analysis of Ca^{2+} and protein

The Ca^{2+} taken up by the membranes was determined by atomic absorption spectroscopy in a Perkin–Elmer spectrophotometer, Model 305, as described previously[8]. The protein was measured by the method of Lowry *et al.*[19] using bovine plasma albumin as a standard.

Reagents

All the chemical reagents were analytical grade. Ruthenium red was obtained from B.D.H.

RESULTS

Effect of ruthenium red on passive Ca^{2+} binding by sarcoplasmic reticulum

Isolated sacroplasmic reticulum binds Ca^{2+} and other cations in the absence of ATP. This binding in the absence of ATP, we have designated as passive binding as opposed to Ca^{2+} uptake in the presence of ATP[8]. However, it should be noted that most of the Ca^{2+} taken up in the presence of ATP also remains bound by the membranes of the sarcoplasmic reticulum if a precipitating agent, such as oxalate, is not present. We have designated this binding of Ca^{2+} in the presence of ATP as "active binding"[8].

Previous work from our laboratory[9] showed that the Ca^{2+} binding capacity

of sarcoplasmic reticulum was decreased by about 50% after the extraction of the lipid fraction of the reticulum. This observation led us to conclude that the lipids, probably the phospholipids, are normally responsible for a relatively large fraction of the Ca^{2+} binding by intact sarcoplasmic reticulum, although other workers have reported no contribution from the phospholipids to Ca^{2+} binding[26]. The explanation for these differences have been discussed elsewhere[9].

In the present study, we determined the effect of ruthenium red on the Ca^{2+} binding by intact and lipid-free sarcoplasmic reticulum to investigate the contribution of phospholipids to the binding of the fraction of Ca^{2+} which is sensitive to ruthenium red.

Fig. 1 shows that 25 μM of ruthenium red decreases the passive Ca^{2+} binding by intact membranes from the value of 140 nmoles/mg of protein to about 75 nmoles/mg of protein, but the lowest concentration of ruthenium red employed (10 μM) already has a significant effect on the Ca^{2+} binding by sarcoplasmic reticulum. The inhibitor effect of ruthenium red is also observed even after extracting the lipid fraction of the membranes. Before the addition of ruthenium red the reticulum retained 140 nmoles of Ca^{2+}/mg of protein or 50 nmoles of Ca^{2+}/mg of protein if the lipids had been removed. The addition of 100 μM of ruthenium red decreased these values of Ca^{2+} binding to about 50 nmoles and 6 nmoles per mg of protein, respectively.

Thus, it is apparent from these results that it is the fraction of Ca^{2+} bound by the proteins which is significantly affected by ruthenium red. As the calsequesterin appears to be the main protein responsible for the binding of Ca^{2+} (ref. 20), probably it is the principal component which apparently is sensitive to ruthenium red.

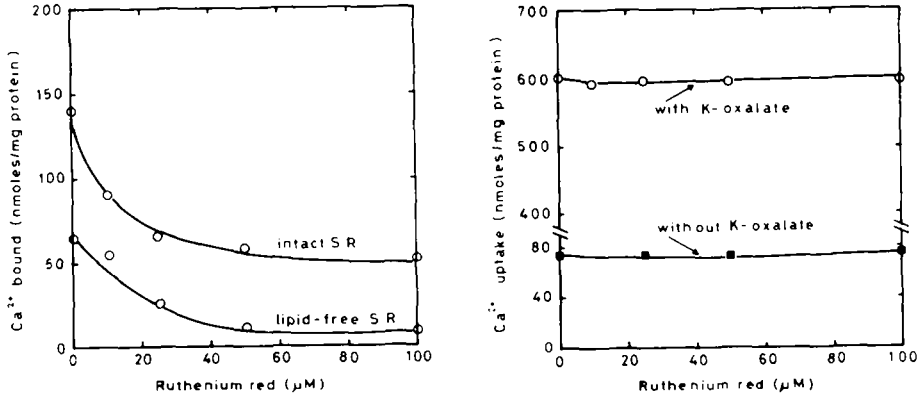

Fig. 1. Effect of ruthenium red on passive Ca^{2+} binding by intact and lipid-free sarcoplasmic reticulum (S.R.) The reaction medium contained 10 mM imidazole (pH 7.0), 3 mM $CaCl_2$ and 2.0 mg of protein in a final volume of 10 ml. The equilibration period was 5 min at room temperature (25 °C). Upper curve, intact sarcoplasmic reticulum; lower curve, lipid-free sarcoplasmic reticulum.

Fig. 2. Effect of ruthenium red on active Ca^{2+} uptake by isolated sarcoplasmic reticulum in the presence and in the absence of potassium oxalate (K-oxalate) The reaction medium contained 10 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 5 mM ATP and 2.0 mg of protein in a final volume of 10 ml. When potassium oxalate was present, its concentration was 5 mM. The equilibration period was 10 min at room temperature (25 °C). ■- -■, without potassium oxalate; ○—○, with potassium oxalate.

Effect of ruthenium red on the active Ca^{2+} uptake by sarcoplasmic reticulum

The effect of ruthenium red was tested in two types of active uptake of Ca^{2+} by sarcoplasmic reticulum. In one case, the active uptake was studied at room temperature in the absence of potassium oxalate and in the presence of 5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 5 mM ATP at a value of 7.0 regulated by 10 mM imidazole. Under these conditions the active uptake of Ca^{2+} varies between 60 and 80 nmoles/mg of protein (Fig. 2).

Ruthenium red in concentrations up to 100 μM does not affect the uptake of Ca^{2+} by sarcoplasmic reticulum. The same insensitivity to ruthenium red is observed when the uptake of Ca^{2+} takes place in the presence of 5 mM potassium oxalate which greatly increases the retention of Ca^{2+} by sarcoplasmic reticulum[1]. Fig. 2 shows that about 600 nmoles of Ca^{2+} per mg of protein are retained in the absence of ruthenium red and concentrations of the dye up to 100 μM do not affect significantly the amount of Ca^{2+} retained.

Since active uptake of Ca^{2+} is not sensitive to the presence of ruthenium red which depresses the passive binding (Fig. 1), it occurred to us that a likely explanation for the different effect of this compound on the retention of Ca^{2+} by sarcoplasmic reticulum was that ATP, present during active uptake of Ca^{2+} by reticulum, complexed the ruthenium red so that this substance could not interact with the membranes of the reticulum. Therefore, we investigated whether ATP would act as a protective agent against the interaction of ruthenium red with the membranes. Fig. 3 shows that indeed 5 mM ATP significantly prevents the binding of ruthenium red to the sarcoplasmic reticulum membranes.

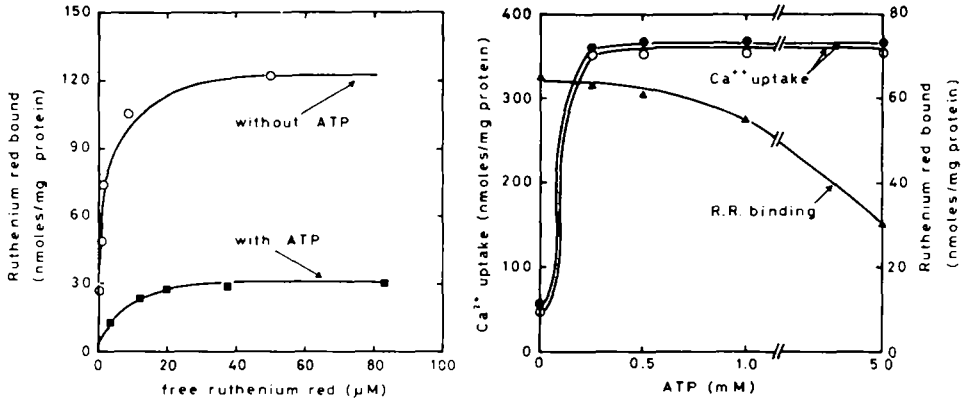

Fig. 3. Effect of ATP on the binding of ruthenium red to the isolated sarcoplasmic reticulum membranes. The reaction medium contained 2. 0 mg of protein and the following concentrations of ruthenium red: 10, 20, 30, 50 and 100 μM in a final volume of 5 ml. When ATP was present, its concentration was 5 mM. The equilibration period was 5 min at room temperature (25 °C). ○—○, without ATP; ■—■, with ATP.

Fig. 4. Effect of ruthenium red (R.R.) on active Ca^{2-} uptake by isolated sarcoplasmic reticulum when the binding of ruthenium red to the membranes is changed by several concentrations of ATP. The reaction medium contained 10 mM imidazole (pH 7.0), 5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 5 mM potassium oxalate, 40 μM of ruthenium red (if present) and 2.0 mg of protein in a final volume of 5 ml. The concentrations of ATP are indicated on the abcissa. The equilibration period was 10 min at room temperature (25 °C). ▲—▲, ruthenium red binding; ○—○, Ca^{2+} uptake in presence of ruthenium red; ●—●, Ca^{2+} uptake in absence of ruthenium red.

In the absence of ATP, sarcoplasmic reticulum binds maximally about 120 nmoles of ruthenium red per mg of protein, and this value is depressed to about 30 nmoles per mg of protein when 5 mM ATP is present (Fig. 3).

This protector effect of ATP against the interaction of ruthenium red with the membranes, at first appeared to be responsible for preventing ruthenium red from acting on the active Ca^{2+} uptake mechanism of the sarcoplasmic reticulum. However, we find that ruthenium red has no effect on the active Ca^{2+} uptake, under conditions of low ATP which do not affect the binding of the dye to the membranes. Fig. 4 shows that 0.25 mM of ATP is sufficient to induce active uptake of Ca^{2+} (in the presence of potassium oxalate), and, that at this concentration, ATP does not prevent the binding of ruthenium red to the membranes. Thus, we observed that when the concentration of ATP is 0.25 mM, there is already maximal Ca^{2+} uptake at about 360 nmoles of Ca^{2+}/mg of protein, even though the ruthenium red bound is not affected by this low concentration of ATP. The level of Ca^{2+} uptake is the same whether ruthenium red is present or absent (Fig. 4). Thus, the lack of effect of ruthenium red on the active Ca^{2+} uptake has to be explained in terms other than a complexing of the ruthenium red by ATP.

Effect of ruthenium red on the Ca^{2+}-dependent ATPase

Since Ca^{2+}-dependent ATPase has been extensively described as a component of the active Ca^{2+} uptake system of the sarcoplasmic reticulum[2-7], we tested the effect of ruthenium red on its activity.

The Mg^{2+}-ATPase was differentiated from the $(Mg^{2+}+Ca^{2+})$-ATPase by the presence of EGTA without adding Ca^{2+} to the medium. The effect of ruthenium red on these ATPase activities was tested in absence and in presence of potassium oxalate. Fig. 5 shows that in the absence of potassium oxalate, Mg^{2+}-ATPase is not affected by ruthenium red, which significantly inhibits the Ca^{2+}-stimulated ATPase.

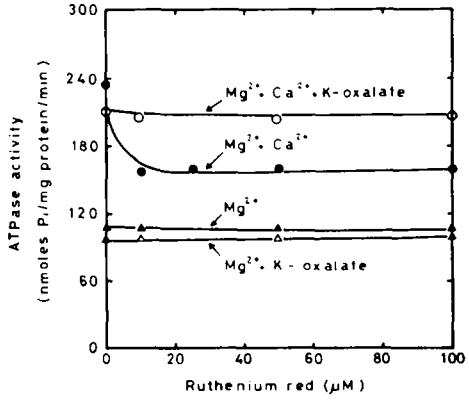

Fig. 5. Effect of ruthenium red on the Mg^{2+}-ATPase and $(Mg^{2+}+Ca^{2+})$-ATPase of the isolated sarcoplasmic reticulum in presence and in absence of potassium oxalate (K-oxalate). The reaction mixture contained 10 mM imidazole (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, 2.5 mg of protein and 5 mM ATP in a final volume of 5 ml. When potassium oxalate was present, its concentration was 5 mM. The equilibration period was 10 min at room temperature (25 °C). ▲—▲, Without Ca^{2+} and potassium oxalate; ●—●, without potassium oxalate; △—△, without Ca^{2+}, with potassium oxalate; ○—○, with potassium oxalate.

At a concentration of 10 μM of ruthenium red the activity of Ca^{2+}-stimulated ATPase is inhibited about 60% of its value measured in absence of the dye, and preliminary experiments showed that the Ca^{2+}-dependent ATPase is sensitive to the inhibitory effect of ruthenium red even at lower concentrations. On the other hand, if potassium oxalate is present, ruthenium red does not inhibit either the Ca^{2+}-dependent ATPase or the Mg^{2+}-ATPase. Furthermore, it is noted that Ca^{2+}-stimulated ATPase is slightly inhibited by the potassium oxalate itself (Fig. 5). In spite of this lower level of initial ATPase activity, ruthenium red does not lower the activity to the values observed in the absence of potassium oxalate (Fig. 5).

It is difficult to visualize how the oxalate functions to prevent the action of ruthenium red on the Ca^{2+}-ATPase of sarcoplasmic reticulum. The fact that ruthenium red does not inhibit the Ca^{2+} transport, but inhibits the ATPase in the absence of oxalate means that either the two processes can be dissociated or that the remaining ATPase activity is sufficient to promote the level of Ca^{2+} transport observed.

DISCUSSION

The significant finding reported here is that ruthenium red, while it inhibits the passive binding, it has no effect on the active uptake of Ca^{2+} by isolated sarcoplasmic reticulum. This observation is rather intriguing particularly if we think of the active uptake as a process which depends on the interaction of Ca^{2+} with specific binding sites of the reticulum membranes before it is transported. However, those sites responsible for the transport of Ca^{2+} probably are relatively few, and, evidently, are not sensitive to ruthenium red, while the bulk of the binding sites of the membrane are non-specific and interact with ruthenium red. On the other hand, if ATP acts on the membrane to increase the affinity of the binding sites for Ca^{2+} (ref. 8), this effect of ATP apparently remains intact even in the presence of ruthenium red.

Therefore, since in the presence of oxalate ruthenium red does also not inhibit the accumulation of Ca^{2+} by sarcoplasmic reticulum which in this case is not bound to the membranes, but is in a precipitated form, it is presumed that ruthenium red does not affect the Ca^{2+} pump mechanism itself.

Ruthenium red has been described as being specific for mucopolysaccharides[15] and, in fact, the binding of Ca^{2+} by glycoproteins isolated from the mitochondrial membranes is sensitive to this dye[21,22,24]. However, this specificity of ruthenium red is not well documented, and we find that dye binds to sarcoplasmic reticulum and inhibits the passive binding of Ca^{2+}, most of which has been postulated to be bound by calsequestrin, a protein recently described by MacLennan and Wong[20] and which has a high capacity for Ca^{2+}.

Since ATP prevents the binding of ruthenium red to the membranes (Fig. 3), we thought that active Ca^{2+} uptake was not inhibited by the dye due to the presence of ATP. However, we verified that a concentration of ATP at which the binding of ruthenium red to the membranes was not affected, but which was enough to permit Ca^{2+} uptake (in the presence of potassium oxalate), ruthenium red remained ineffective in inhibiting active Ca^{2+} uptake. Thus, the insensitivity of the active Ca^{2+} uptake mechanism to the dye must be explained in terms other than formation of an inactive complex with ATP.

These results with sarcoplasmic reticulum are in contrast to those reported

for isolated rat liver mitochondria[10,11]. In this system, ruthenium red, at concentrations even lower than those employed in these studies, inhibited the transport of Ca^{2+} as well as the high and low affinity binding of Ca^{2+} by the mitochondrial membranes[10,11].

Ruthenium red has already proved useful in studies with skinned muscle fibers performed to determine whether mitochondria play a role in regulating the concentration of intracellular free Ca^{2+} (ref. 12). Gillis[12] showed that ruthenium red slows down the speed of relaxation of red muscle fibers which are rich in mitochondria, but has no effect on the fibers where sarcoplasmic reticulum is well developed, and normally regulates the concentration of intracellular free Ca^{2+}.

Rossi *et al.*[23] proposed that ruthenium red added before Ca^{2+} transport into mitochondria is complete, inhibits its accumulation, by competing with Ca^{2+} for the same binding sites, possible Ca^{2+} carrier molecules. However, if the dye is added after transport of Ca^{2+} is complete, ruthenium red does not induce its release probably because the Ca^{2+} have reached other binding sites not available to ruthenium red.

The effect of ruthenium red on the active Ca^{2+} uptake by mitochondria suggests that there is a carbohydrate involved in the mechanism of Ca^{2+} translocation into mitochondria[11]. In fact, glycoproteins have been isolated from the intermembrane space of rat liver mitochondria[21] and from the inner membrane of ox liver mitochondria[22,24] that bind Ca^{2+} firmly[21] and this Ca^{2+} binding is significantly inhibited by ruthenium red [22,24].

The different behavior of sarcoplasmic reticulum and mitochondria is probably due to the different nature of the Ca^{2+} carrier in these two organelles. While in mitochondria the Ca^{2+} carrier apparently is a glycoprotein[11,14,21–24], in sarcoplasmic reticulum it has been assumed that it is the ATPase itself that functions as a Ca^{2+} carrier[6,25].

We tested the effect of ruthenium red on the Mg^{2+}-ATPase and ($Mg^{2+} + Ca^{2+}$)-ATPase and found that, as in mitochondria[11] and in red blood cell membranes[16], the Mg^{2+}-ATPase in sarcoplasmic reticulum is not affected by ruthenium red. On the other hand, the Ca^{2+}-stimulated ATPase in inhibited by about 60% by the dye if potassium oxalate is absent. Thus, ruthenium red causes an uncoupling of Ca^{2+} uptake and Ca^{2+}-ATPase activity when oxalate is not present. Very recently, McFarland and Chan[27] also showed that increasing pH, freezing and aging can uncouple extra ATPase and Ca^{2+} uptake activities but only in absence of oxalate. This fact led them to conclude that oxalate changes the mechanism of those processes.

The high sensitivity of the Ca^{2+} transport mechanism of mitochondria to ruthenium red and the lack of effect of this substance on the active uptake of Ca^{2+} by sarcoplasmic reticulum may prove to be a useful tool in determining the function of the two cell organelles in regulating muscular activity. However, it should be kept in mind that the passive binding of Ca^{2+} by sarcoplasmic reticulum is sensitive to ruthenium red and that this fraction of Ca^{2+} may also play a role in contraction[8,9].

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