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Interaction of Ruthenium Red with Isolated Sarcolemma

Vítor M. C. Madeira and M. C. Antunes-Madeira

Department of Zoology, University of Coimbra, Portugal

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Summary. The stain ruthenium red binds very strongly to isolated sarcolemma and the maximal binding is about 125 nmoles/mg protein, decreasing slightly in lipid-extracted membranes. The binding is half maximal when the free stain concentration is about 1.0 μM, for both intact and lipid-depleted material. The nucleotide, ATP, reduces markedly the binding and the apparent affinity of the membranes for the stain. Minute concentrations of ruthenium red (10 μM) inhibit by 80 to 90% the Ca^{++}-binding by sarcolemma. The inhibition does not depend on the Ca^{++} concentration and is similar in both intact and lipid-extracted preparations. Ruthenium red inhibits the ATPase activity of sarcolemma. The inhibition is decreased by increasing the ATP concentration in the medium.

The primary events of excitation-contraction coupling in muscle occur at the plasma membrane in the sarcolemma [27, 28]. Much work has been done to clarify the mechanism which links the electrical activity of the plasma membrane to the contractile machinery inside the cell [15, 29, 30, 35, 37], but the detailed events have not yet been elucidated.

The sarcoplasmic reticulum is the main muscular structure in skeletal muscle which regulates the intracellular Ca^{++} concentration [11, 12], and therefore, the contractile activity [43, 44]. However, in some types of muscle, which are relatively poor in sarcoplasmic reticulum, other structures may work as Ca^{++} regulators, such as mitochondria [5, 16] and, possibly, sarcolemma [1]. A fraction of Ca^{++} involved in the contraction-relaxation cycle may enter or leave the muscle cell through sarcolemma [24, 27, 32].

Furthermore, we must necessarily consider an energetic efflux of Ca^{++} through the sarcolemma to maintain a constant intracellular concentration of Ca^{++} since the concentration of free Ca^{++} in the extracellular medium is much greater than that inside the muscle cell [23, 25, 32]. Although sarcoplasmic reticulum [6, 11, 12, 44] and possibly mitochondria [5, 16, 18] may account for most Ca^{++} sequestration during relaxation of the muscle cell, there is a constant invasion of the cell by an inward net diffusion of

Ca^{++} which must be constantly pumped to the outside. The free energy compensation for such a transport may be derived from ATP which can be hydrolyzed by a Ca^{++}-ATPase present in sarcolemma [10, 11, 23, 34].

In recent investigations the Ca^{++} binding sites of the sarcolemma were characterized [17, 22, 38]. In the present study we have investigated the effect of ruthenium red on the Ca^{++} binding and on ATPase activity of sarcolemma. The results revealed that both activities are strongly inhibited by ruthenium red. Therefore, ruthenium red may constitute a useful tool for studying sarcolemmal phenomena which may be implicated in the regulation of muscle activity, particularly since the stain does not affect extensively the binding of Ca^{++} by sarcoplasmic reticulum [39].

Materials and Methods

Isolation of the Biological Material

Fragmented sarcolemma was isolated from rabbit skeletal muscles as described elsewhere [19, 22]. Sarcolemmal membranes were exhaustively washed in 0.6 M KCl and de-ionized water to remove actomyosin. The material seems free from other subcellular fractions as revealed by phase-contrast and electron microscopy [19, 20]. The existence of a particular Ca^{++}-ATPase different from that of myofibrils and fragmented sarcoplasmic reticulum [22] gave us confidence that the preparations are quite pure. Succinate dehydrogenase activity [3] was not found and, therefore, it is assumed that there are no mitochondria contaminating the sarcolemmal preparations.

Protein was determined by the biuret method [9] after dissolving the membranes in 30% KOH. Bovine serum albumin was used as standard.

Binding of Cations

The binding of cations was studied as described previously [22] in media at pH 7.0 containing 4 mM imidazole and substances under test in the desired concentrations.

Binding of Ruthenium Red

Binding of ruthenium red was determined at pH 7.0 and room temperature by incubating, for 10 min, 1.0 mg of sarcolemmal protein in media (total volume 5 ml) containing 4 mM imidazole, ruthenium red and other substances under test in the desired concentrations. The suspensions were centrifuged at $4{,}000 \times g$ for 10 min. The supernatants were saved and free ruthenium red was determined at 540 nm. The amount of ruthenium red bound to the sedimented protein was determined by the difference between the concentration of ruthenium red originally present and that after the incubation with protein. The controls without protein included all the reagents present in samples.

Lipid Extraction

Lipid was extracted in aqueous acetone-ammonia as described by Fleischer and Fleischer [14]. Lipid phosphorus was estimated following the method of Schneider [31] modified by Carvalho and Leo [6] and inorganic phosphate was determined in solutions of the digested residues by the method of Chen, Toribara and Warner [8].

ATPase Activity

ATPase activity was determined in media (total volume 5 ml), containing 5 mM Tris (pH 7.4), 30 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$ and various ATP concentrations. Reactions proceeded until no more than 10 to 20% of the total ATP was hydrolyzed. The reaction was stopped by adding 1 ml of 20% TCA. Suspensions were filtered through Whatman paper No. 41 and inorganic phosphate was analyzed in the filtrates by the method described by Taussky and Shorr [36].

Reagents

All the reagents used were of biochemical analyses grade. Salts were obtained from Merck; ATP, Tris and imidazole from Sigma; ruthenium red from BDH.

Results

Effect of Ruthenium Red on the Ca^{++} Binding by Isolated Sarcolemma

The stain ruthenium red inhibits very strongly the binding of Ca^{++} by isolated sarcolemma as depicted in Fig. 1. Ruthenium red inhibits the

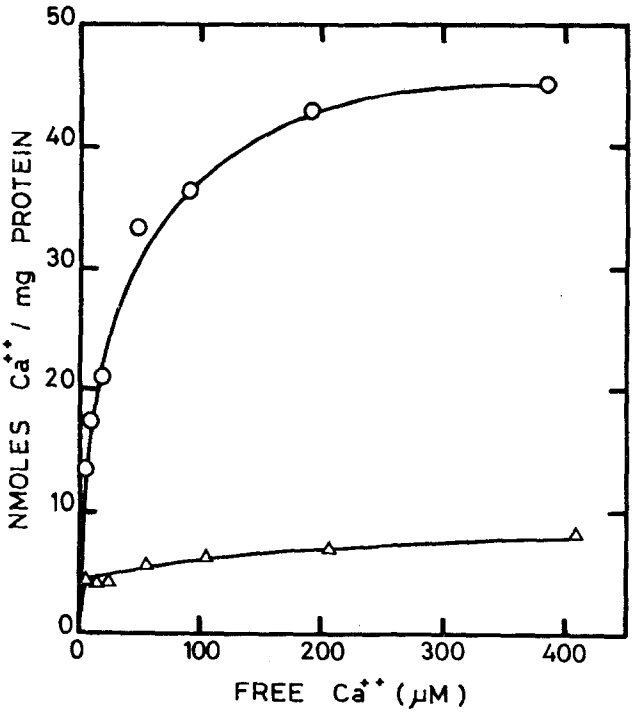

Fig. 1. Saturation curves for Ca^{++} binding by sarcolemma in absence (○) and in presence (△) of 20 μM ruthenium red. In the abscissa we represent the free Ca^{++} concentration in equilibrium with the complex sarcolemma-Ca. Ca^{++} does not reverse the inhibition of ruthenium red on Ca^{++} binding

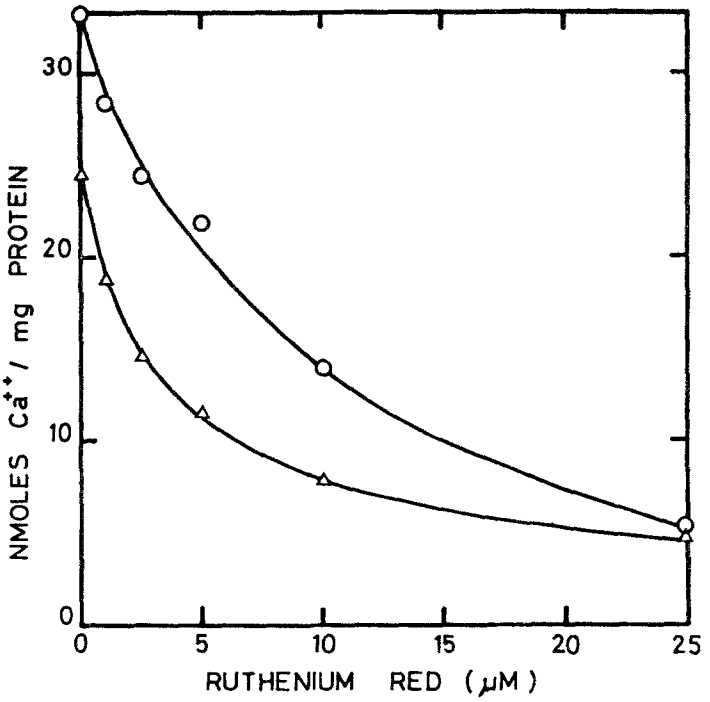

Fig. 2. Effect of ruthenium red on Ca^{++} binding by intact (○) and lipid-extracted sarcolemma (△). Lipid extraction was performed in aqueous acetone-ammonia solutions. The amount of phospholipid phosphorus extracted was about 70%. The medium (pH 7.0) contained 4 mM imidazole and 100 μM $CaCl_2$

binding by about 80 to 90% when added in concentrations of 10 to 20 μM. Concentrations above 20 μM inhibit the binding as much as 90%. The extent of inhibition does not depend appreciably on the Ca^{++} concentration in the medium (Fig. 1). These results indicate that the mechanism of interaction of ruthenium red with fragmented membranes cannot be explained by competition between Ca^{++} and the stain for the same binding sites.

We made considerable effort to characterize the inhibition of the Ca^{++} binding by ruthenium red. However, the classical reciprocal plots and other similar treatments failed to give us clear-cut information about the type of inhibition.

Ruthenium red inhibits the binding in intact as well as in lipid-extracted preparations. The stain in concentration of 6 to 7 μM inhibits the Ca^{++} binding by about 50% in both preparations (Fig. 2).

Binding of Ruthenium Red by Sarcolemma

The maximal binding of ruthenium red by sarcolemma is of the order of 120 to 140 nmoles/mg protein (Fig. 3). The maximal binding decreased

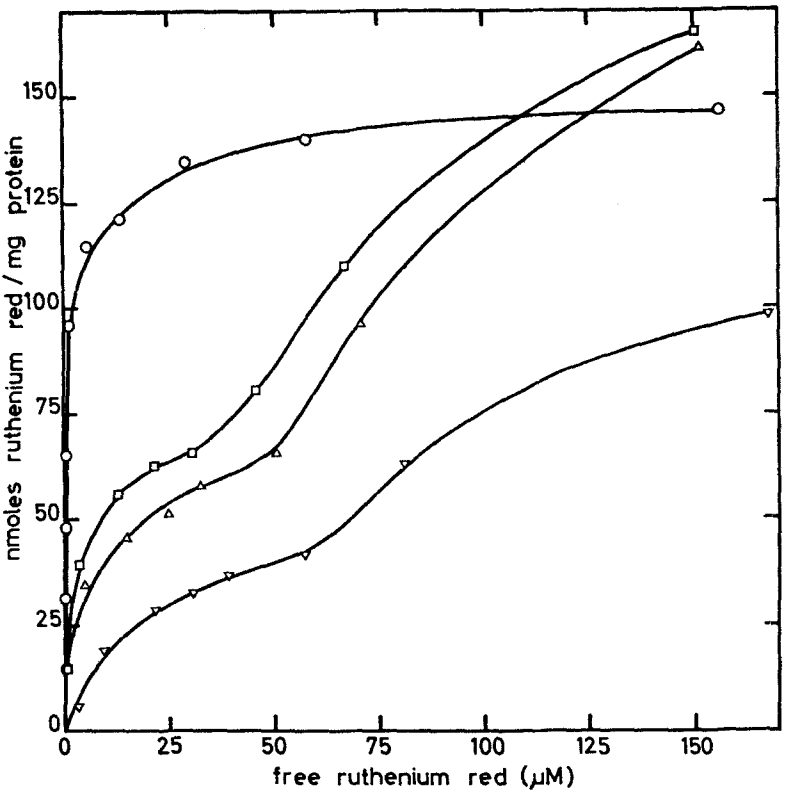

Fig. 3. Effect of ATP on the binding of ruthenium red by sarcolemma. ATP considerably decreases the binding for free ruthenium red concentrations up to about 50 μM. Symbols are: ○, control; □, 0.5; △, 1.0; ▽, 2.0 mM ATP

slightly in acetone-treated preparation and the apparent dissociation constant of the complex membrane-stain is about 0.8 to 1.2 μM for both intact and lipid-extracted preparations.

ATP inhibits the binding of ruthenium red by isolated sarcolemma (Fig. 3). ATP is effective for free ruthenium red concentrations up to about 50 μM. The nucleotide lowers the apparent affinity of sarcolemma for ruthenium red. The apparent dissociation constants are 48, 55 and 100 μM when ATP is present at concentrations of 0.5, 1.0 and 2.0 mM, respectively. Furthermore, ATP (0.5 mM) abolishes by about 30% the inhibition of Ca^{++} binding by ruthenium red.

Effect of Ruthenium Red on the ATPase Activity of Sarcolemma

As depicted in Fig. 4, ruthenium red inhibits significantly the ATPase activity of fragmented sarcolemma. The inhibition depends on the ATP

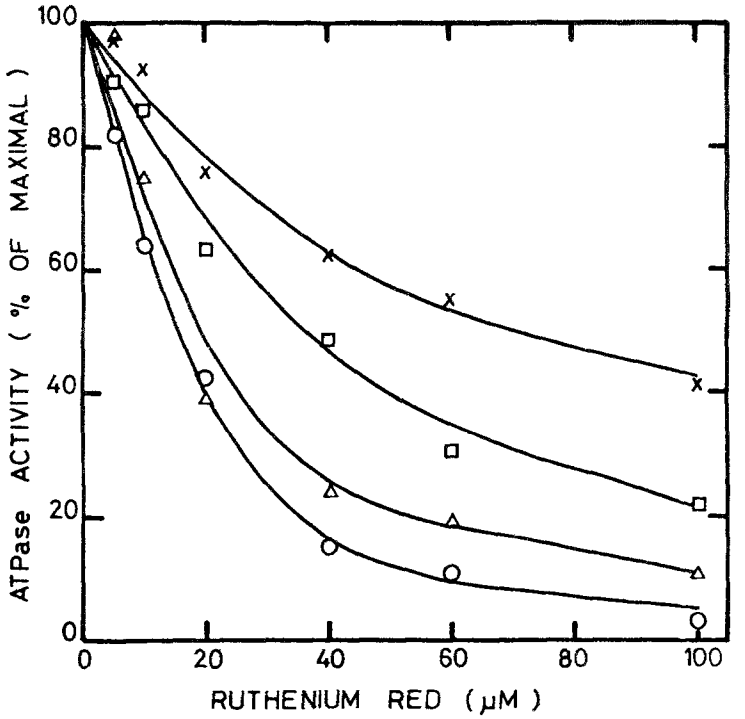

Fig. 4. Effect of ATP concentration upon the inhibition of sarcolemmal ATPase activity by ruthenium red. Media (pH 7.4) contained 5 mM Tris, 30 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, ATP and ruthenium red as desired. Symbols are: ×, 2.0; □, 1.0; △, 0.4; ○, 0.2 mM ATP

concentration which affects markedly the inhibition pattern. The nucleotide protects against the inhibitory effect of ruthenium red.

The inhibition varied from 85 to 35% (40 μM of added stain), for ATP concentrations ranging from 0.2 to 2.0 mM.

Discussion

Effect of Ruthenium Red on Ca^{++} Binding

As described in the Results, ruthenium red inhibits the Ca^{++} binding by sarcolemma. The inhibition does not depend on the Ca^{++} concentration in the medium and may represent irreversible conformational changes of the membrane macromolecules which cause the loss of their Ca^{++} binding activity.

It can be inferred that the main components of the sarcolemma which are affected by ruthenium red are proteins, although the present results do

not exclude that phospholipids may also be affected. Thus, ruthenium red inhibits the Ca^{++} binding both in intact and lipid-extracted preparations, but the main components which bind ruthenium red must be proteins since the capacity and affinity of sarcolemma for the stain are not appreciably affected by removal of the lipid fraction. There is some evidence that ruthenium red binds to sarcolemmal proteic material with high content in aspartic and glutamic residues [40].

The sarcolemmal phospholipids probably do not contribute significantly to the binding of Ca^{++}, although studies with acetone extractions seem to indicate the contrary, since sarcolemma extracted with acetone binds less Ca^{++} [22]. This decrease of Ca^{++} binding is not observed when the sarcolemmal lipids are removed by phospholipase A-treatment followed by washing with albumin to remove the digestion products (*unpublished observations*). Furthermore, the phospholipids present in sarcolemma [13, 20] do not bind Ca^{++} [2].

ATP prevents the binding of ruthenium red as clearly shown in Fig. 3, for concentrations of the stain up to about 50 μM. This effect of ATP may be explained in two different ways: (1) ATP may chelate some of the ruthenium red, thus lowering the effective free stain concentration in the medium, or (2) ATP may act by lowering the affinity of sarcolemmal membranes for the stain. The data of Fig. 3 do not permit us to decide between these two possibilities.

The nucleotide has no effect on the binding of ruthenium red for concentrations of the stain above 50 μM (Fig. 3). The explanation for this finding is not clear at the moment.

Effect of Ruthenium Red on Sarcolemmal ATPase

The inhibition of the ATPase activity may represent alteration of the enzyme conformation. However, the stain may also prevent the active site of the enzyme from interacting with Ca^{++}, since its activity is stimulated by Ca^{++} [22]. The inhibition of ATPase by ruthenium red is apparently prevented by increasing the ATP concentration on the medium which also prevents the binding of ruthenium red (Figs. 3 and 4).

Physiological Significance of the Study

Ruthenium red inhibits the binding of Ca^{++} by organelles and molecules which may be implicated in Ca^{++} transfer, such as mitochondria [4, 26, 41], mitochondrial [33] and cartilage [42] proteins, and other structures [7, 21].

However, no effect was observed on the ATP-dependent Ca^{++} uptake by muscle sarcoplasmic reticulum, although ruthenium red may inhibit some of its passive Ca^{++} binding [7, 39]. Gillis [16] has shown that ruthenium red inhibits relaxation of certain stripped muscle fibers poor in sarcoplasmic reticulum, and this fact was interpreted in terms of inhibition of Ca^{++} transport by mitochondria. These types of results do not exclude that sarcolemma is also involved in Ca^{++} regulation inside those and other types of muscle fibers. Therefore, if ruthenium red also inhibits the Ca^{++} transport in sarcolemma, the stain may be useful, in future studies, for determining whether the movements of Ca^{++} through sarcolemma are involved in the regulation of the contractile activity of the muscle cell, since the stain does not affect markedly the Ca^{++} uptake and binding by sarcoplasmic reticulum [7, 39].

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