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Ruthenium red affects the intrinsic fluorescence of the calcium-ATPase of skeletal sarcoplasmic reticulum

Marie-Jo Moutin, Catherine Rapin and Yves Dupont

Laboratoire de Biophysique Moléculaire et Cellulaire, URA 520 du CNRS, DBMS / LBIO, Centre d'Etudes Nucléaires de Grenoble, Grenoble (France)

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We have studied the effect of Ruthenium red on the sarcoplasmic reticulum Ca^{2+}-ATPase. Ruthenium red does not modify the Ca^{2+} pumping activity of the enzyme, despite its interaction with cationic binding sites on sarcoplasmic reticulum vesicles. Two pools of binding sites were distinguished. One pool (10 nmol/mg) is dependent upon the presence of micromolar Ca^{2+} and may therefore represent the high-affinity Ca^{2+} transport sites of the Ca^{2+}-ATPase. However, Ruthenium red only slightly competes with Ca^{2+} on these sites. The other pool (15–17 nmol/mg) is characterized as low-affinity cation binding sites of sarcoplasmic reticulum, distinct from the Mg^{2+} site involved in the ATP binding to the Ca^{2+}-ATPase. The interaction of Ruthenium red with these low-affinity cation binding sites, which may be located either on the Ca^{2+}-ATPase or on surrounding lipids, decreases tryptophan fluorescence level of the protein. As much as 25% of the tryptophan fluorescence of the Ca^{2+}-ATPase is quenched by Ruthenium red (with a dissociation constant of 100 nM), tryptophan residues located near the bilayer being preferentially affected.

Introduction

Ruthenium red (RR) is a synthetic dye which has been extensively used for electron microscopy of cells and tissues [1]. Typically, this polycationic compound ($[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]^{6+}$) reacts with polyanions [1,2].

For a long time, RR has been known to affect Ca^{2+} fluxes across membranes. It inhibits the mitochondrial Ca^{2+} uptake [3,4] and release [5]. It inhibits a Ca^{2+}-uniport into chloroplasts [6] and the smooth-muscle plasma membrane Ca^{2+} pump [7]. Several authors [8–11] have described a stimulatory effect of RR on the Ca^{2+} sequestration by sarcoplasmic reticulum (SR) vesicles from skeletal and cardiac muscle. No direct effect of the dye on the Ca^{2+}-dependent ATPase activity responsible for the Ca^{2+} accumulation within the SR was, however, observed [9]. The increased net Ca^{2+} sequestration, which was markedly higher on junctional SR vesicles than on non-junctional ones, was attributed to the blockade of the SR Ca^{2+} releasing channel of these junctional regions. The purified calcium release channel (or ryanodine receptor) of SR is indeed highly sensitive to RR [12–14].

However, very recently, RR was reported to stain several Ca^{2+} binding proteins on SDS-polyacrylamide gels, including the SR Ca^{2+}-ATPase [15]. In this paper we report that RR interacts with Ca^{2+} and Mg^{2+} sites of SR vesicles, displaying high affinity for these sites. We observed a decrease of 25% of the intrinsic fluorescence of the Ca^{2+}-ATPase as a consequence of RR binding to low-affinity cation binding sites located either on the protein or on surrounding lipids.

Materials and Methods

Preparation of SR vesicles. Crude SR preparation was made from rabbit skeletal muscle as described in Ref. 16. No sucrose gradient step was performed. The vesicles (around 20–30 mg of protein/ml) were suspended in 300 mM sucrose, 20 mM Mops-Tris at pH 7.2 and frozen in liquid nitrogen where they were stored until used. Protein concentration was determined spectrophotometrically at 280 nm in the pres-

Abbreviations: SR, sarcoplasmic reticulum; RR, Ruthenium red; Mops, 3-(*N*-morpholino)propane sulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Correspondence: M.-J. Moutin, Laboratoire de Biophysique Moléculaire et Cellulaire, URA 520 du CNRS, DBMS/LBIO, Centre d'Etudes Nucléaires de Grenoble, BP 85 X, 38041 Grenoble, France.

ence of 1% sodium dodecyl sulfate as described in Ref. 17.

Solutions. Free Ca^{2+} concentrations were established using EGTA and calculated with the association constants given by Vianna [18]. The contaminating Ca^{2+} was supposed to be 10 μM.

RR was purchased from Sigma. RR concentrations in stock solutions were verified spectrophotometrocally by the method described by Luft [1].

$^{45}Ca^{2+}$ transport measurements by rapid filtration. Experiments were carried out using the Rapid-Filtration System (Bio-Logic Co, Claix, France) with nitrocellulose filters (Millipore, 0.65 μm) at room temperature (20–22°C). SR vesicles (5 mg/ml) were first equilibrated in 20 mM $MgCl_2$, 100 mM KCl, 20 mM Mops at pH 7.2. Then 0.45 mg/ml were preincubated in 50 μM $^{45}CaCl_2$ (0.2 μCi/ml), 20 mM $MgCl_2$ in the same buffer. The enzyme, which has bound $^{45}Ca^{2+}$, was applied to a filter. 15 s after the beginning of the preincubation, the 'uptake solution' containing 50 μM $^{45}CaCl_2$ (0.2 μCi/ml), 20 mM $MgCl_2$ and 5 mM Mg-ATP in the same buffer was passed through the enzyme-loaded filter using the Rapid Filtration apparatus.

Data were corrected with blanks obtained for each condition by omitting ATP in the uptake solution. During experiments performed in the presence of RR, 20 μM RR were added in the preincubation and uptake media.

Measurements of RR binding to SR vesicles. Stoddart et al. [19] have shown that an increase of the extinction maximum of RR absorbtion accompanies the mixing of the dye with polygalacturonic acid. This effect was caused by the interaction of RR with the polysaccharide. We have observed similar spectral shifts when mixing RR with SR vesicles. This finding permits us to obtain very simple measurements of RR binding to SR vesicles. RR binding was estimated to be the difference between optical densities at 560 and at 510 nm.

Experiments were performed at 30°C with a Lambda 5 Perkin Elmer spectrophotometer. Reaction media were composed of various SR protein concentrations in 75 mM [illegible] 40 mM Tris (pH 7.2). Spectra were corrected by substracting the signal given by the SR vesicles incubated in the absence of RR.

In order to correlate the spectroscopic data with a more direct assay some experiments (binding capacities with varying Mg^{2+} concentrations) were performed as follows. RR binding was measured after incubation of the SR vesicles with the dye, removal of the membranes by centrifugation and spectroscopic assay of the RR concentration remaining in the supernatant. We obtained similar results with two different methods (not shown).

Measurements of $^{45}Ca^{2+}$ binding to SR Ca^{2+}-ATPase. Experiments were carried out at room temperature using nitrocellulose filter (Millipore, 0.45 μm). SR vesicles (5 mg/ml) were first equilibrated in 75 mM Mops, 40 mM Tris at pH 7.2. Then 0.5 mg of SR proteins were incubated with the 'Ca^{2+} binding medium' containing 75 mM Mops, 40 mM Tris (pH 7.2), 100 μM Ca^{2+} and various EGTA concentrations to obtain the various free Ca^{2+} concentrations. Mg^{2+} and RR were also added as indicated in the legend of the figures. Ca^{2+}-bound enzyme was then applied to a filter using a filter holder connected to a powerful vacuum pump. Reaction was stopped at 30 s by applying vacuum to immediately eliminate all $^{45}Ca^{2+}$ in excess. Data were corrected with blanks obtained for each condition by omitting the SR vesicles.

Measurements of Ca^{2+}-ATPase intrinsic fluorescence. Measurements were carried out with a Modular Optical System (MOS-1000 from Bio-Logic Co., Claix, France) comprising the following elements: (1) a 1 × 1 cm cuvette holder and propeller stirrer permitting sample injection in microliter quantities with Hamilton syringes without opening the cuvette holder; (2) a light source with a 150 W Xe(Hg) lamp and a grating monochromator set at 297 nm; (3) a first detection channel using a head-on photomultiplier set at 90° from the incident light. The emitted light was filtered by a combination of a cut-off filter and a black filter, transmitting the light between 320 and 400 nm; (4) a second detection channel measuring part of the incoming light which was deflected by a beam-splitter installed before the cuvette holder. It was used to compensate the effect of the lamp fluctuations on the fluorescence intensity.

Experiments were performed with 2.5 or 25 μg of SR proteins/ml in media containing 75 mM Mops, 40 mM Tris (pH 7.2).

Measurement of [^{14}C]ATP binding to Ca^{2+}-ATPase. Experiments were carried out at room temperature in the absence of Ca^{2+} in 75 mM Mops and 40 mM Tris at pH 7.2. SR vesicles (9 or 18 mg/ml) were first equilibrated in 0.5 mM EGTA. Then 50 μl (0.45 or 0.9 mg of SR proteins) were added to 1 ml of the 'ATP binding medium' containing varying ATP concentrations (0.01–2 μCi/ml), 0.5 mM EGTA, 0 or 5 mM $MgCl_2$ and 0 or 20 μM RR. After 30 s incubation in ATP, 0.95 ml of the solution (0.4 or 0.8 mg of ATP-bound enzyme) were rapidly filtered through a 0.45 μm Millipore filter to eliminate [^{14}C]ATP in excess. Data were corrected with blanks obtained for each condition by filtering the same solution but without proteins.

Results

Lack of effect of RR on the rate of Ca^{2+} transport by SR vesicles

In Fig. 1 we have represented the initial phase of Ca^{2+} uptake by SR vesicles measured by rapid filtra-

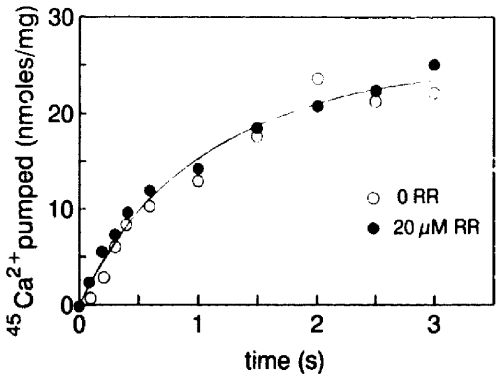

Fig. 1. Effect of RR on the Ca^{2+} uptake by SR vesicles. Ca^{2+} uptake was measured by Rapid-Filtration as described in Materials and Methods in the presence of 50 μM $^{45}Ca^{2+}$, 20 mM Mg^{2+}, 5 mM Mg-ATP, 100 mM KCl, 20 mM Mops.K (pH 7.2) and 0 (○) or 20 μM RR (●).

tion in the presence of 50 μM $^{45}Ca^{2+}$, 5 mM Mg-ATP and 20 mM Mg^{2+} at pH 7.2 in Mops-KCl buffer. No calcium ionophore or calcium oxalate was added. The experiment was performed either in the absence or in the presence of 20 μM RR. In the absence of RR, SR vesicles were able to accumulate 24 nmol of Ca^{2+} per milligram of SR proteins within 3 s. During the first 300 ms. Ca^{2+} accumulated at a constant rate of 1.3 $\mu mol \cdot mg^{-1} \cdot min^{-1}$. The addition of 20 μM RR did not modify this initial rate of Ca^{2+} transport.

This experiment demonstrates that RR does not affect the rate of the Ca^{2+} pumping by the SR Ca^{2+}-ATPase. It is in agreement with results previously reported by Seiler et al. [9] explaining that RR does not modify the Ca^{2+}-dependent ATPase activity of SR. The SR Ca^{2+}-ATPase function is not sensitive to RR, in contrast to that of several other Ca^{2+} transporters [3,6,7].

Determination of the RR binding sites on SR vesicles

In order to determine the sites for RR on SR vesicles, we have used the metachromic effect that occurred on mixture of the dye with the vesicles (see Materials and Methods).

In Fig. 2 we have represented a titration experiment of the RR binding sites on SR vesicles. The optical signals (quantified by the difference between the optical densities at 560 and 510 nm) were obtained by mixing increasing concentrations of RR with various concentrations of SR proteins. Experimental media contained 200 μM EGTA, no magnesium, 40 mM Tris and 75 mM Mops (pH 7.2). Using the results obtained with 0.1 or 0.3 mg of SR proteins/ml, we determined the concentration of sites (B_{max}) of RR on SR vesicles to be around 25 nmol of RR bound/mg of SR proteins (0.1 mg/ml of proteins bound a maximum of 2.4 μM RR; 0.3 mg/ml bound a maximum of 7.5 μM).

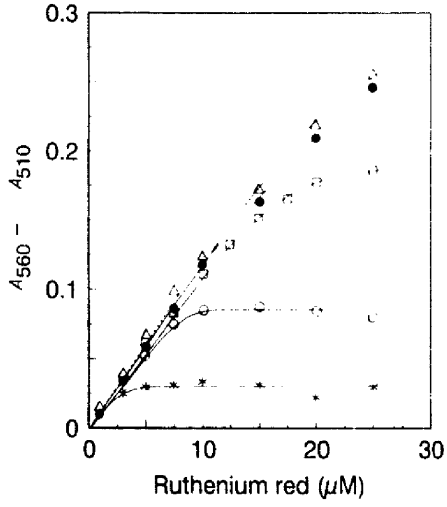

Fig. 2. Measurement of RR binding to SR vesicles. RR binding was measured using the difference between the absorbance (A) at 560 and at 510 nm (see Materials and Methods). Experiments were performed at 30°C in the presence of 200 μM EGTA, 75 mM Mops and 40 mM Tris at pH 7.2. RR binding was evaluated for the following SR protein concentration: (*) 0.1 mg/ml, (○) 0.3 mg/ml, (□) 0.6 mg/ml, (●) 1 mg/ml and (△) 3 mg/ml. The dashed line represents the assymptotic value where all RR is bound.

We show in Fig. 3 the Scatchard plot of RR binding to SR vesicles at 0.6 mg/ml. This figure also illustrates the effect of adding 20 mM Mg^{2+} and also the effect of adding 100 μM Ca^{2+}. The Scatchard representation of the RR binding in the same conditions as in Fig. 2 (200 μM EGTA and 0 Mg^{2+}) is linear and gives a B_{max} of 27 nmol/mg. The apparent dissociation constant (K_d) of the RR for its SR sites is around 0.7 μM. The replacement of 200 μM EGTA by 100 μM Ca^{2+} (0 Mg^{2+} and 100 μM Ca^{2+}) greatly reduces the observed B_{max}. It decreases from 27 to 17 nmol of RR bound/mg of SR proteins, the K_d being also reduced to 0.3 μM.

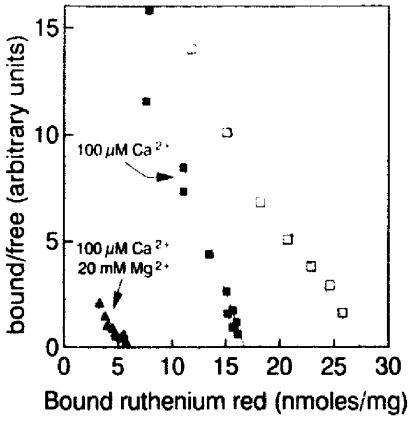

Fig. 3. Scatchard plot of RR binding to SR vesicles. Experiments were performed as described in the legend to Fig. 2 with 0.6 mg/ml of SR proteins, at pH 7.2 (75 mM Mops, 40 mM Tris) in the presence of either (□) 200 μM EGTA and no Mg^{2+} or (■) 100 μM Ca^{2+} and no Mg^{2+} or (▲) 100 μM Ca^{2+} and 20 mM Mg^{2+}.

When, in addition, 20 mM Mg^{2+} was added we observed a further decrease of the B_{max}, which was now reduced to 6 nmol/mg The K_d of the remaining sites was around 0.9 μM.

In Fig. 4, we have represented the effect of adding increasing concentrations of Mg^{2+} on the level of RR binding to SR vesicles (at 0.6 mg/ml) incubated with 100 μM Ca^{2+} in Mops-Tris buffer at pH 7.2. The level of RR binding decreased continuously above 3 mM Mg^{2+} and it can be estimated that around 1 M Mg^{2+} may be required to displace most of the RR from its SR vesicles sites. Assuming a K_d of 5 mM for Mg^{2+} this gives a very high affinity of RR for these sites with a dissociation around 0.1 μM. Similar results were obtained when adding millimolar concentrations of Ca^{2+} instead of Mg^{2+} (not shown).

Therefore, two types of RR binding site were found on SR vesicles. The first pool, representing 8–10 nmol per milligram of SR protein, is sensitive to Ca^{2+} at micromolar concentrations. The second, 17–19 nmol/mg, is unaffected by the presence of micromolar Ca^{2+} but sensitive to millimolar Mg^{2+} or Ca^{2+}. RR has submicromolar affinity constants for the two types of site. These sites are largely widespread on the SR vesicles. Thus they may be located on very abundant structures of this membranous preparation.

Considering the highly charged structure of RR and as its binding was evaluated rapidly after adding the dye, RR access to SR intraluminal sites (like the Ca^{2+}-binding protein calsequestrine) should have been limited.

As they are dependent upon the presence of micromolar Ca^{2+}, the sites of the first pool may be the high-affinity Ca^{2+} transport sites of the Ca^{2+}-ATPase. In order to verify this hypothesis we have then studied, by the use of a filtration method, the effect of RR on the Ca^{2+} binding to the SR Ca^{2+}-ATPase.

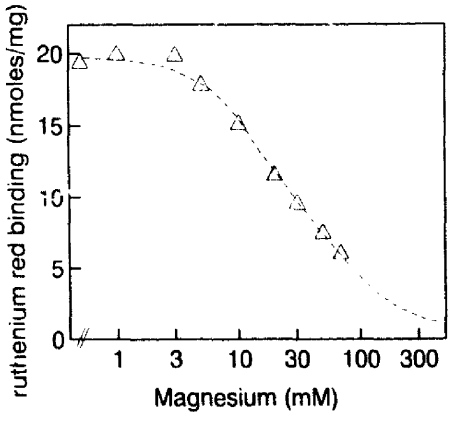

Fig. 4. Effect of Mg^{2+} on the level of RR binding to SR vesicles. Experiments were performed as described in the legend to Fig. 2 with 0.6 mg/ml of SR proteins, at pH 7.2 (75 mM Mops, 40 mM Tris) in the presence of varying Mg^{2+} concentration.

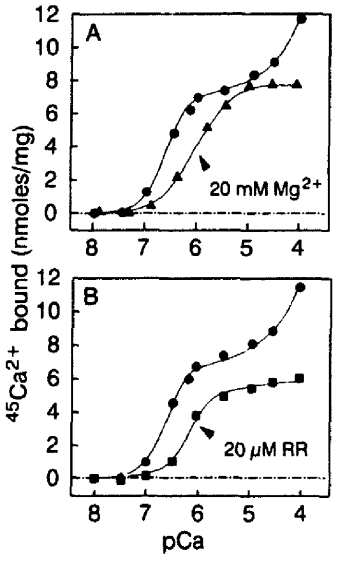

Fig. 5. Measurements of Ca^{2+} binding to SR vesicles in the presence of 20 mM Mg^{2+} and 20 μM RR. Experiments were performed by filtration as described in Materials and Methods at pH 7.2 (75 mM Mops, 40 mM Tris) in the presence of 100 μM $^{2+}Ca^{2+}$ and various EGTA concentrations to obtain the indicated pCa. (●) 0 Mg^{2+} and 0 RR, (▲) 20 mM Mg^{2+}, (■) 20 μM RR.

Effect of RR on the Ca^{2+} binding by SR vesicles

In Fig. 5 we have represented the Ca^{2+} binding to SR vesicles (0.5 mg/ml) as a function of the Ca^{2+} concentration in various conditions: 0 Mg^{2+} and 0 RR, 20 mM Mg^{2+} or 20 μM RR. Experimental media contained Mops-Tris buffer at pH 7.2.

In the absence of Mg^{2+} and RR, two types of Ca^{2+} site were observed on SR vesicles. The first type (B_{max} = 8 nmol/mg) presents a high affinity for Ca^{2+}, with a K_d around 0.5 μM. It represents the high-affinity Ca^{2+} transport sites of the SR Ca^{2+}-ATPase [20,21]. Low-affinity Ca^{2+} sites are also observed at higher Ca^{2+} concentrations (greater than 10 μM). Their B_{max} and K_d cannot be determined accurately.

The presence of 20 mM Mg^{2+} greatly modifies the Ca^{2+} binding curve. We observe a disappearance of the low-affinity Ca^{2+} binding sites, showing that these sites are not very specific for Ca^{2+}, and may be occupied as well with Mg^{2+}. A shift to low values of pCa of the first part of the curve was also observed. The affinity for Ca^{2+} of the high-affinity sites is reduced from 0.5 to 2 μM, no change of the B_{max} can be observed. This effect reveals a competition between Ca^{2+} and Mg^{2+} on the high-affinity Ca^{2+} binding sites. The competitive effect of Mg^{2+} for Ca^{2+} on these sites of the enzyme was previously described [20].

The presence of 20 μM RR also greatly changes the shape of the Ca^{2+} binding curve. At the highest Ca^{2+} concentrations (10–100 μM) the binding is reduced by RR from 12 to 6 nmol of Ca^{2+} bound per milligram of

SR proteins. Like Mg^{2+}, RR is therefore able to bind to low-affinity divalent cationic sites of SR vesicles. This was already shown here by our measurements of RR spectral shifts (Fig. 3).

Addition of 20 μM RR also produces a shift of the first part of the Ca^{2+} binding curve to lower pCa again showing a competition between the dye and Ca^{2+} on the high-affinity binding sites.

The high-affinity Ca^{2+} binding that we measured may be safely attributed to the SR Ca^{2+}-ATPase, since there is no other abundant protein with high-affinity sites for Ca^{2+} known within the SR preparation. RR appears therefore to be a competitive inhibitor of Ca^{2+} on the high-affinity Ca^{2+} transport sites of this enzyme. However, no significant consequence was seen on the Ca^{2+} pumping activity in standard conditions (Fig. 1).

Effect of RR on the intrinsic fluorescence of the Ca^{2+}-ATPase

In Fig. 6 we show the effect on the intrinsic fluorescence level of the SR Ca^{2+}-ATPase (at 2.5 μg/ml) induced by adding increasing concentrations of RR. The experiment was performed with 200 μM EGTA in the absence or in the presence of 200 mM Mg^{2+}.

With 200 mM Mg^{2+}, a small decrease of the fluorescence level was observed when the RR concentration was increased. Adding more Mg^{2+} did not reduce further the decrease produced by RR. This decrease may be due to an inner filter effect of the dye since the logarithm of the fluorescence intensity decreases linearly as a function of the RR concentration.

In the absence of Mg^{2+}, RR produced a severe decrease of the fluorescence level. At each RR concentration we have calculated the difference between the effect measured in the presence and in the absence of Mg^{2+}. We have then represented this difference in

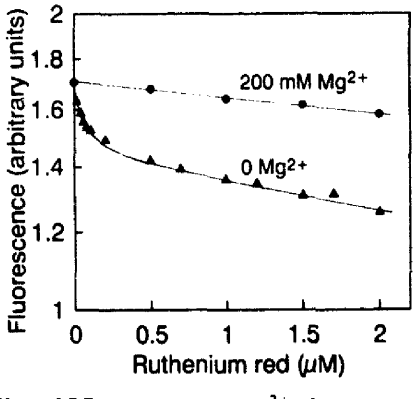

Fig. 6. Effect of RR on the level of Ca^{2+}-ATPase intrinsic fluorescence in the absence and in the presence of 200 mM Mg^{2+}. Fluorescence was measured as described in Materials and Methods at pH 7.2 (75 mM Mops, 40 mM Tris) with 2.5 μg/ml of SR proteins, 200 μM EGTA and 0 (▲) or (●) 200 mM Mg^{2+}.

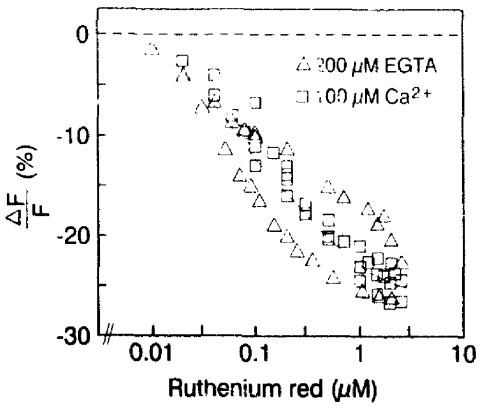

Fig. 7. Decrease of the level of intrinsic fluorescence of the SR Ca^{2+}-ATPase with or without free Ca^{2+} in the medium. Experiments were performed as described in the legend to Fig. 6 at pH 7.2 (75 mM Mops and 40 mM Tris) with 2.5 μg/ml of SR proteins and (△) 200 μM EGTA or (□) 100 μM free Ca^{2+}. In these both conditions, the difference between the effects obtained without and with 200 mM Mg^{2+} were calculated and plotted as percent of the signal observed with Mg^{2+}.

percent of the signal observed with Mg^{2+} in Fig. 7. Experiments were performed either in the presence of 200 μM EGTA or in the presence of 100 μM Ca^{2+}. In both conditions, we observed a 25% decrease of the fluorescence level of the SR Ca^{2+}-ATPase when adding RR. The K_d for RR of the sites involved in this effect ranges between 60 and 120 nM. It should be noted that we performed the experiment with 2.5 μg/ml of SR proteins only (corresponding to 15–20 nM of Ca^{2+}-ATPase) in order to be in appropriate conditions to measure this high affinity. This can explain the rather low sensitivity of the experiment.

Therefore, we show here, for the first time, that RR is able to affect drastically the fluorescence emission by the tryptophan residues of the SR Ca^{2+}-ATPase. We propose that the quenching observed is due to the proximity of the bound RR molecules and the tryptophan residues. Luft [1] measured the absorption spectra of solution of RR purified by crystallization. The major peak for RR is at 533 nm but a second significant peak is present at 360 nm. The overlap with the tryptophan fluorescence spectra is thus sufficient to explain that part of the fluorescence energy may be absorbed by RR if it binds close to tryptophan residues. The dye is able to quench as much as 25% of the intrinsic fluorescence level of this protein in the absence of Mg^{2+} as shown in Fig 7.

The RR binding sites involved in this effect are not the high-affinity Ca^{2+} sites of the SR Ca^{2+}-ATPase since the intrinsic fluorescence level of the enzyme shows the same decrease when adding RR in the absence and in the presence of 100 μM Ca^{2+}. However, they may be the RR binding sites characterized as low-affinity divalent cationic (Mg^{2+} or Ca^{2+}) sites in

previous paragraphs. The presence of at least 200 mM Mg^{2+} is needed to completely inhibit this extinction by RR which shows an apparent affinity constant of approximatly 0.1 μM. The K_d for Mg^{2+} on these sites is therefore in the millimolar range.

We were then interested in localizing the Mg^{2+}-sensitive RR binding sites involved in the effect observed on the Ca^{2+}-ATPase intrinsic fluorescence. Two types of experiments were performed. Firstly, we have investigated the effect of RR on the intrinsic fluorescence level of SR vesicles previously treated with the ionophore A23187. This should allow us to distinguish between two types of trytophan residues within the protein [22–26]. Secondly, we have examined the effect of RR on the binding of [^{14}C]ATP to the enzyme, since it is well known that Mg^{2+} is involved in this step [27].

Effect of A_{23187} on the intrinsic fluorescence decrease produced by RR

Most tryptophans of the SR Ca^{2+}-ATPase reside in a hydrophobic environment [22–24]. The liposoluble calcium ionophore A23187 quenches a very large proportion of the total intrinsic fluorescence of the Ca^{2+}-ATPase of SR vesicles preparation [25,26]. This effect was attributed to the quenching of 11 of the 13 tryptophan residues of the enzyme that are supposed to be located within the portion of the protein located either near the protein/lipid interface or deeply embedded in the membrane [22,23,26]. Two remaining tryptophan residues are likely to be found in the cytoplasmic part of the protein: W50 and W552. One of them, W552, is probably located very close to the ATP binding site [22,23]. It is well known that the SR Ca^{2+}-ATPase contains a Mg^{2+} binding site (with millimolar dissociation constant) which is involved in the ATP binding to the enzyme [27,28].

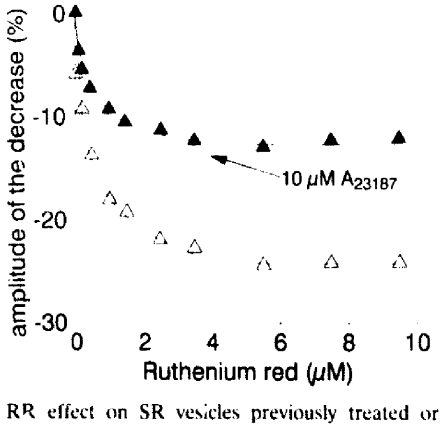

Fig. 8. RR effect on SR vesicles previously treated or not with A_{23187}. Experiments were performed as described in the legend to Fig. 6 at pH 7.2 (75 mM Mops and 40 mM Tris) with 25 μg/ml of SR proteins which were previously treated (▲) or not (△) with 10 μM A23187. In these both conditions, the difference between the effects obtained without and with 200 mM Mg^{2+} were calculated and plotted as percent of the signal observed with Mg^{2+}.

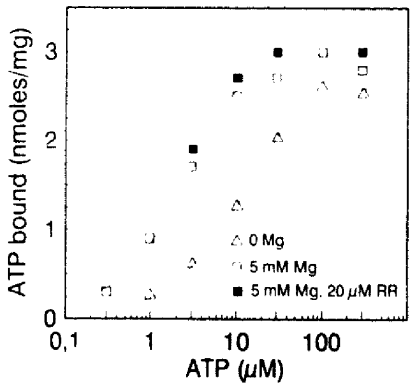

Fig. 9. RR effect on the [^{14}C]ATP binding to the SR Ca^{2+}-ATPase. Experiments were performed by filtration as described in Materials and Methods at pH 7.2 (40 mM Tris and 75 mM Mops) in the 'absence' of free Ca^{2+} (0.5 mM EGTA) and with (△) 0 Mg^{2+} and 0 RR, or (□) 5 mM Mg^{2+}, or (■) 5 mM Mg^{2+} and 20 μM RR.

In Fig. 8 we have represented the percentage of Mg^{2+}-sensitive intrinsic fluorescence decrease as a function of RR concentration on SR vesicles (25 μg/ml) which were incubated or not with 10 μM A23187. The experiments were performed with 200 μM EGTA in Mops-Tris buffer at pH 7.2. The addition of 10 μM A23187 produced a rapid decrease of the SR vesicles intrinsic fluorescence of 60–70% either in the absence or in the presence of 200 mM Mg^{2+}. As for experiments of Fig. 7, we have calculated the difference between the effect of RR obtained in the presence and in the absence of Mg^{2+} and plotted it as percent of the signal observed with Mg^{2+}. At maximal RR concentrations, 24% of the intrinsic fluorescence of non-treated vesicles are quenched in a Mg^{2+}-sensitive manner whereas, in the same conditions, only 13% of the intrinsic fluorescence of A23187-treated vesicles are quenched. From this result we can conclude that RR affects preferentially the A23187-sensitive tryptophan residues that are situated in the transmembrane portion of the ATPase rather than those which are not susceptible to the ionophore. It is thus unlikely that the Mg^{2+}-sensitive RR binding sites are situated close to the ATP binding site

Effect of RR on the binding of [^{14}C]ATP by the Ca^{2+}-ATPase

Here we directly question whether RR is able to affect the binding of ATP to the Ca^{2+}-ATPase. The binding of [^{14}C]ATP to the enzyme is shown as a function of the ATP concentration in the range of 0.3–300 μM (Fig. 9). The experiments were carried out either in the absence or in the presence of 5 mM Mg^{2+} in Mops-Tris buffer at pH 7.2. It was not possible to analyze accurately experiments performed with RR in

the absence of Mg^{2+}, since part of the [^{14}C]ATP precipitated with the dye, which may yield incorrect results.

In the absence of Mg^{2+}, the SR vesicles bind about 3 nmol of ATP per milligram of SR proteins with a K_d of 15 μM. When 5 mM Mg^{2+} was added, the same amount of ATP binds to the vesicles, but the K_d decreased to 2.5 μM. This demonstrates that Mg^{2+} actively participates in the ATP binding process to the SR Ca^{2+}-ATPase, as previously shown [27,29].

Then 20 μM RR was added during the experiment performed with Mg^{2+} in order to see if RR competes with Mg-ATP binding. Such a RR concentration was largely sufficient, knowing that the binding constant for RR was found around 0.1 μM (Fig. 7). However, no effect of RR was observed. This can be explained as follows: (i) either RR can replace Mg^{2+} and plays the same role as Mg^{2+} in ATP binding, or, (ii) the target of RR binding is not the Mg^{2+} site involved in the ATP binding. The first explanation is rather unlikely, knowing the result obtained with A23187.

Discussion

RR sensitivity has been frequently used as a marker for the Ca^{2+} release channel of junctional SR [30,31]. In this report we investigate the possible effects of this dye on the major component of SR membranes, the Ca^{2+}-ATPase. In agreement with previous reports [9,32], we confirmed that 20 μM RR does not alter the Ca^{2+}-ATPase pumping activity (Fig. 1). Following results demonstrate that submicromolar RR is able to interact with cationic sites of the Ca^{2+}-ATPase and/or of surrounding lipids, and decreases the intrinsic fluorescence level of the protein.

A maximum of 25–27 nmol of RR can bind to 1 mg of SR proteins (Fig. 3). RR binding sites were divided in two pools on the basis of their sensitivity to divalent cations. The first pool may represent the high-affinity Ca^{2+} transport sites of the Ca^{2+}-ATPase for the following reasons: (i) RR binding was dependent upon the presence of micromolar amounts of Ca^{2+} and therefore upon the state of the enzyme (Ca^{2+}-bound or Ca^{2+}-free, Fig. 3); (ii) it is likely that RR is a competitive inhibitor of Ca^{2+} on the Ca^{2+}-ATPase high-affinity sites, as measured by $^{45}Ca^{2+}$ filtration experiments (Fig. 5). The second pool represents non-specific cation binding sites of the SR Ca^{2+}-ATPase or of a closely associated element for the following reasons: (i) RR binding to these sites was inhibited by millimolar amounts of Mg^{2+} (Figs. 3 and 4); (ii) RR was able to inhibit Ca^{2+} binding to low-affinity cation sites of SR vesicles as observed by ^{45}Ca filtration (Fig. 5); (iii) RR produced an instantaneous drastic Mg^{2+}-sensitive decrease of the Ca^{2+}-ATPase intrinsic fluorescence (Figs. 6 and 7).

The striking result of this report is the 25% decrease of the Ca^{2+}-ATPase intrinsic fluorescence observed as a result of RR binding to low-affinity cation binding sites of SR vesicles (with a K_d for RR of 60–120 nM). The observed effect is instantaneous (a few seconds) and should therefore not be the consequence of RR binding to intraluminal sites; RR being highly charged, its diffusion through SR membrane would take more time. The fluorescence quench by RR comes from the proximity of cation binding sites located at the SR membrane surface and of some of the tryptophan residues of the Ca^{2+}-ATPase. These sites may be situated either on the Ca^{2+}-ATPase or on another element of the membrane sufficiently close to the protein to enable quenching of its intrinsic fluorescence as a result of RR binding.

Our primary hypothesis was that the low-affinity cation binding sites may be the Mg^{2+} sites implicated in the ATP binding to the enzyme, which were described by several authors [27,29]. Two kinds of experiments were performed to try to localize the RR-sensitive Mg^{2+} binding sites on SR membranes. Both were in contradiction with an RR effect due to its binding to the Mg^{2+} site involved in the ATP binding. However, we showed that RR preferentially affects the A23187-sensitive tryptophan residues that are proposed to be located in the transmembrane part of the protein (Fig. 8). Up to now the following possibilities remain. RR may bind to low-affinity cation binding sites of the Ca^{2+}-ATPase distinct from that involved in the ATP binding. The existence of other Mg^{2+} sites on the Ca^{2+}-ATPase was proposed by various authors and is still discussed [27,29,33]. Moreover, Girardet et al. [34] have recently demonstrated the existence of cationic binding sites located at a very close distance to tryptophan residues of the enzyme by using tryptophan to terbium fluorescence energy transfer.

RR may also bind to other elements, proteins or lipids, closely associated to the protein. The possibility of an RR attachment to an associated protein having binding sites for divalent cations is unlikely in view of the extensive fluorescence quenching. Moreover, our results of SR vesicle staining on SDS-polyacrylamide gels (not presented) show that exclusively Ca^{2+}-ATPase and intraluminal calsequestrine display staining by RR as recently observed [15]. The occurrence of an A23187-like effect of RR subsequent to interaction of the dye with lipids (probably anionic) should not be excluded. Luft has indeed described RR affinity for most of the phospholipids [1]. A very recent work has shown Ca^{2+}-ATPase association with particular lipids of the SR membrane [35]. These authors described a model with a "highly polar area existing at the lipid–protein interface in the hinge portion of the Brandl et al. model [23]". We can presume that the highly charged RR molecules interact with SR constituents in this

region to produce its effect on the Ca^{2+}-ATPase intrinsic fluorescence. Cations (Mg^{2+} or Ca^{2+}) may influence the RR interaction by modifying the surface charge of the membrane.

Finally, the fact that RR does not affect significantly the Ca^{2+}-ATPase pumping activity is not surprising since RR competes only slightly with the high-affinity Ca^{2+}-binding sites, since it does not seem to bind to the ATP-Mg binding site, but only to low-affinity cation binding sites.

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