Ruthenium red inhibits the mitochondrial Ca^{2+} uptake in intact bovine spermatozoa and increases the cytosolic Ca^{2+} concentration

Fernanda Rigoni and Renzo Deana°

Department of Biology and °*Institute of Biological Chemistry, University of Padova and Unit of Consiglio Nazionale delle Ricerche for the Study of the Physiology of Mitochondria, Via Marzolo 3, 35131 Padova, Italy*

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The uptake and cycling of Ca^{2+} by ejaculated bovine spermatozoa are almost completely abolished by ruthenium red, antimycin A or FCCP. The inhibitory effect of ruthenium red is also observed after washing of the dye-pretreated cells followed by addition of digitonin or filipin. In contrast, the inhibition is overcome by A23187 treatment. It is concluded that ruthenium red penetrates into intact spermatozoa, inhibits the mitochondrial Ca^{2+} uptake 'in situ', and causes the observed increase of the cytosolic free Ca^{2+} concentration.

(Bovine spermatozoa) *Cytosolic Ca^{2+}* *Mitochondria* *Ca^{2+} transport* *Ruthenium red*

1. INTRODUCTION

It is well established that the intracellular Ca^{2+} concentration regulates a wide range of cell functions, e.g. the coordinated and progressive motility of mammalian spermatozoa [1–5]. It has been found that while bovine epididymal spermatozoa take up calcium rapidly, ejaculated spermatozoa are almost incapable of accumulating Ca^{2+} because of a Ca^{2+} transport inhibitor present in the seminal plasma [6–8]. A net influx of Ca^{2+} occurs during the 'in vitro' capacitation of epididymal sperm [4], while a limited entry of Ca^{2+} induced by ionophores accelerates the capacitation and acrosome reactions of spermatozoa of several animal species [9–11].

Although evidence for an ATP-requiring Ca^{2+} pump and an Na^+/Ca^{2+} antiporter in mammalian spermatozoa has been recently reported, the mechanism(s) regulating the intracellular Ca^{2+} concentration has not been fully characterized [5,12–14]. Mitochondria are most likely the major site of cellular Ca^{2+} sequestration [4,15,16].

Here, we show that ruthenium red added to undamaged spermatozoa blocks the Ca^{2+} cycling by inhibiting the mitochondrial Ca^{2+} uptake in situ and causes an increase of the cytosolic Ca^{2+} concentration.

2. MATERIALS AND METHODS

Chemicals were purchased as indicated: silicone DC 550 fluid (Serva); dinonylphthalate (Fluka); quin 2-AM and A23187 (Calbiochem-Behring); filipin (Upjohn); Percoll and Ficoll 400 (Pharmacia); DTPA (Sigma); $^{45}CaCl_2$ (Amersham). Ruthenium red (Sigma) was purified according to Luft [17], while digitonin (Merck) was recrystallized according to Kun et al. [18]. Ionomycin and

° To whom correspondence should be addressed

Abbreviations: DTPA, diethylenetriaminepentaacetic acid; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)-ethylenediamide; RR, ruthenium red; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

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TPEN were kind gifts from Dr T. Pozzan, Padova; all other reagents were of analytical grade.

2.1. *Isolation of bovine spermatozoa*

Artificially collected bovine semen was allowed to liquify at room temperature for 1 h. Spermatozoa were isolated by centrifugation (900 × *g*, 5 min) of 2 ml semen layered onto 2 ml of a high-density medium containing 150 mM NaCl, 6 mM KCl, 1.5 mM KH_2PO_4, 3 mM $NaHCO_3$, 2 mM glucose, 10 mM Na-Hepes (pH 7.4) and 34% (v/v) Percoll (medium density 1.05). The spermatozoa were resuspended in the same medium devoid of Percoll, at a concentration of 1×10^9 cell/ml. Sperm count and motility were measured by the usual microscope techniques, and cell viability by eosin-nigrosin staining [19]. Only samples with viability higher than 90% were used. Some control experiments were carried out with suspensions of progressively motile spermatozoa obtained by an upward migration in a high-density Ficoll-containing medium as described in [20].

2.2. *Ca^{2+} uptake by spermatozoa*

Uptake and cycling of Ca^{2+} were determined by incubating the washed spermatozoa at 30°C and at 3×10^8 cell/ml of the above-reported medium. Ca^{2+} uptake was started by addition of 1.5 mM $CaCl_2$ and Ca^{2+} cycling by addition of 0.1 μCi $^{45}Ca^{2+}$ (1 mCi/μgion, carrier-free). At fixed time intervals 0.3 ml suspension was withdrawn and rapidly mixed with 0.3 ml of a solution containing 120 mM NaCl and 50 mM sodium EGTA. The suspension was then transferred to a microfuge test tube containing 0.3 ml silicone oil/dinonylphthalate mixture (65:35%, v/v) and centrifuged for 2 min at 12000 × *g* in an Eppendorf 5414 microfuge. The aqueous upper layer was discarded and the tube wall washed with 0.8 ml water, which was afterwards withdrawn together with silicone oil. The test tubes were centrifuged as before to remove the last drops of silicone oil. The pellet was resuspended in 0.8 ml water and vigorously mixed before adding 0.2 ml of 50% (w/v) perchloric acid. Aliquots of the supernatants obtained after centrifugation at 12000 × *g* for 3 min were used for the measurement of radioactivity accumulated in the spermatozoa and the total calcium content with a scintillator counter (Beckman LS 1800) and an atomic absorption spectrophotometer (Perkin-Elmer 305B), respectively.

2.3. *Measurement of cytosolic free Ca^{2+}*

Determination of the cytosolic Ca^{2+} concentration was carried out with the fluorescent indicator quin 2, essentially according to Tsien et al. [21]. Briefly: the spermatozoa, further washed with the basal medium supplemented with 2 mM glucose, 1.5 mM $MgCl_2$, 1.6 mM $CaCl_2$, 0.1 mM EGTA and 0.2 M urea, were finally resuspended in the latter medium, devoid of urea, at 1×10^8 cell/ml and at 36.8°C. 3 μl of 10 mM quin 2 acetoxymethyl ester (quin 2/AM) per ml incubation mixture were added. After 20 min the cells were diluted 3-fold with the same medium and incubated for 2 h. This procedure gave the best results concerning quin 2/AM cellular loading and hydrolysis. The cells were spun down, washed by resuspension in the above-reported medium supplemented with 1 mg/ml of bovine serum albumin and finally resuspended at 20×10^6 cell/ml.

Quin 2 fluorescence was measured with a Perkin-Elmer LS5 spectrofluorimeter with 390 nm excitation and 492 nm emission wavelengths; calibration was carried out as described by Tsien et al. [21]. Occasionally, calibration was also performed by quenching the fluorescence with $MnCl_2$ as reported by Rink et al. [22]. Corrections of fluorescence due to cellular dye leakage were assessed with the membrane-impermeant heavy metal chelator DTPA as described by Rink and Pozzan [23], while the fluorescence quenching due to intracellular heavy metals was corrected by addition of the membrane-permeant heavy metal chelator TPEN [24].

3. RESULTS

Washed ejaculated bovine spermatozoa, resuspended in a Ca^{2+}-free medium, take up Ca^{2+} when this ion is added to the incubation mixture (fig.1). If carrier-free $^{45}Ca^{2+}$ is added to the medium, at the end of the net uptake of calcium, intracellular accumulation of radioactivity takes place, indicating Ca^{2+} cycling and radioisotopic distribution. Addition of ruthenium red to the medium prior to calcium addition causes a complete inhibition of both non-radioactive and la-

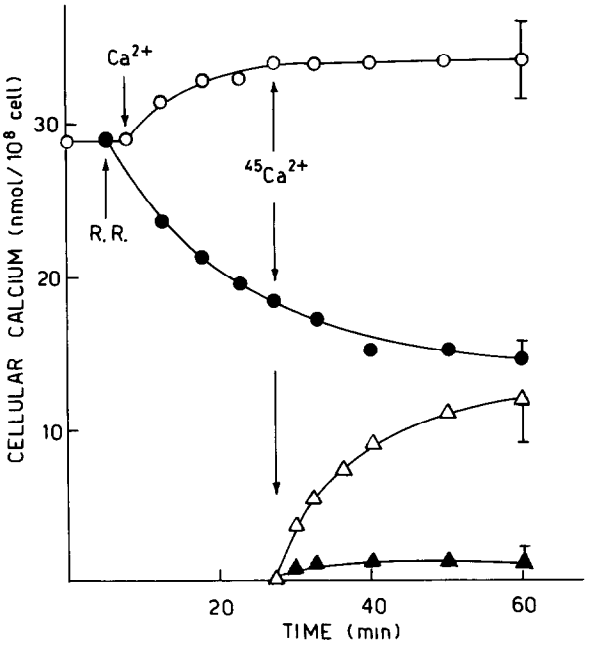

Fig.1. Time courses of Ca^{2+} uptake and cycling by ejaculated bovine spermatozoa in the absence and presence of ruthenium red (R.R.). Values are means ± SD of 5 experiments and represent total calcium (○, ●) and radioactive $^{45}Ca^{2+}$ (△, ▲) content of spermatozoa incubated in the absence (open symbols) or presence (closed symbols) of ruthenium red. 1.5 mM Ca^{2+}, trace amounts of $^{45}Ca^{2+}$ and 5 μM ruthenium red were added at the arrows; other experimental details are given in section 2.

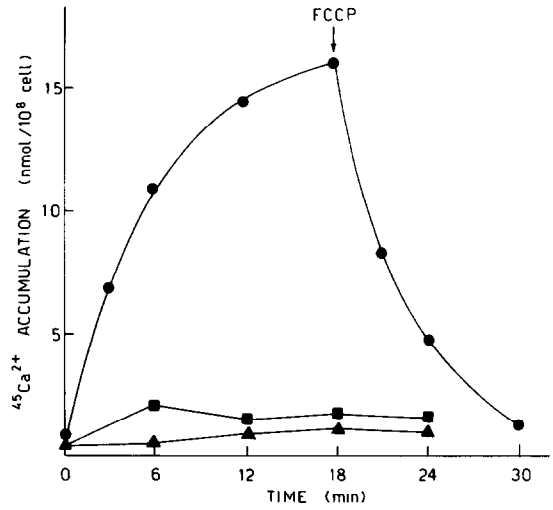

Fig.2. Effect of antimycin A and FCCP on $^{45}Ca^{2+}$ accumulation by bovine spermatozoa. Incubations were started by addition of 1.5 mM $^{45}CaCl_2$ with no other addition (●), after 5 min preincubation with 10 μM rotenone plus 1 μM antimycin A (■) or 2 μM FCCP (▲). Points are means of 3 different experiments.

belled Ca^{2+} influx parallel to an efflux of endogenous calcium.

Evidence that Ca^{2+} taken up by spermatozoa is mainly accumulated in the mitochondria is given in fig.2 where the inhibition of cellular Ca^{2+} uptake by the respiratory chain inhibitor antimycin A and by the uncoupler FCCP is shown. Moreover, addition of FCCP to $^{45}Ca^{2+}$-loaded spermatozoa causes a rapid release of the $^{45}Ca^{2+}$ accumulated.

Ruthenium red has long been used as a histochemical stain for membrane surface mucopolysaccharides and is a well-known inhibitor of calcium uptake by isolated mitochondria [17,25,26]. To ensure that the observed effect of ruthenium red was not due to an inhibition of Ca^{2+} uptake by medium-exposed mitochondria of damaged cells, we repeated the same experiments and obtained similar results, with spermatozoa isolated by active upward migration into a high-density medium [20]. More than 85% of these spermatozoa were progressively motile and at least 98% were found to be viable by the eosin-nigrosin staining technique [19]. Fig.3 shows that while addition of the detergent digitonin to control spermatozoa causes a clear enhancement of $^{45}Ca^{2+}$ uptake, the addition of digitonin to a suspension of ruthenium red-pretreated spermatozoa does not overcome the dye inhibition of ^{45}Ca uptake. Similar results were obtained with filipin (fig.2), a polyene antibiotic which selectively binds and modifies the sperm plasma membranes [27,28], and by hypotonic treatment (not shown).

In contrast, the calcium ionophore A23187, which permeabilizes both cellular and mitochondrial membranes, causes an increase, although to a different extent, of Ca^{2+} uptake in both control and ruthenium red-pretreated spermatozoa.

In our opinion these results indicate that ruthenium red specifically inhibits the mitochondrial Ca^{2+} uptake in situ.

We have measured the cytosolic Ca^{2+} concentration in bovine spermatozoa by means of quin 2 and found that it is normally 105 ± 15 nM.

Quin 2 fluorescence is partially quenched by ruthenium red. Although the calibration for the

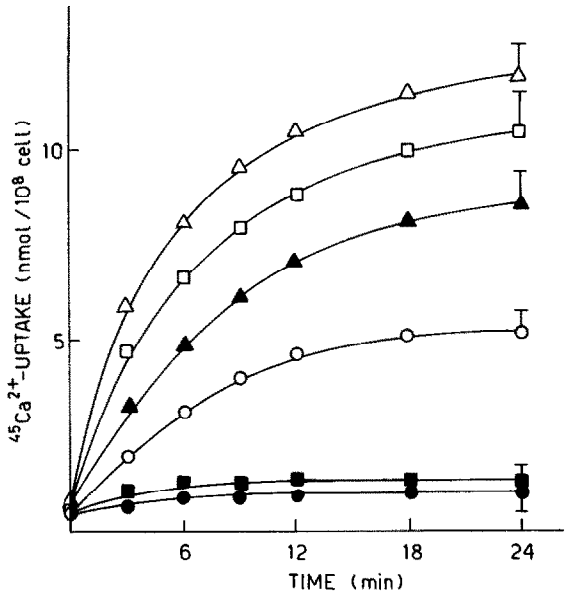

Fig.3. $^{45}Ca^{2+}$ uptake by ruthenium red-pretreated spermatozoa incubated with membrane-permeabilizing compounds. Spermatozoa were incubated for 5 min in the basal medium containing 5 μM ruthenium red, centrifuged for 8 min at 750 × g, washed and resuspended in ruthenium red-free medium supplemented with 0.25 mM Ca^{2+}. After 30 min of equilibration of Ca^{2+} distribution, $^{45}Ca^{2+}$ (carrier-free) was added together with none (○, ●), 500 μM digitonin or 300 μM filipin (□, ■), or 10 μM A23187 (△, ▲). Ruthenium red-pretreated spermatozoa, closed symbols; controls without ruthenium red, open symbols. Points are means ± SD of 5 experiments.

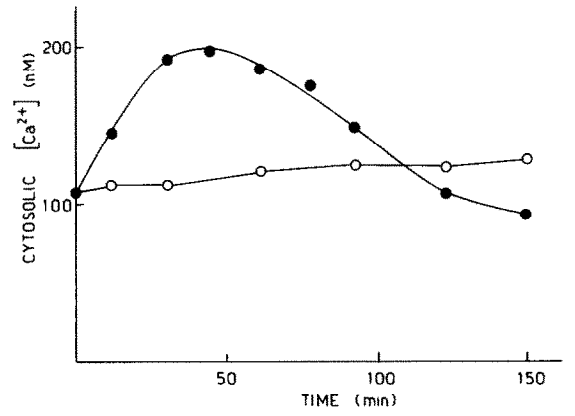

Fig.4. Cytosolic Ca^{2+} concentration of ruthenium red-treated spermatozoa. Ruthenium red was added at the beginning of incubation; points are means of 4 experiments. Control (○) and in the presence of 5 μM ruthenium red (●).

assessment of free Ca^{2+} was possible even in the presence of the dye, we usually washed out ruthenium red after incubation with quin 2-loaded spermatozoa and then performed the measurement. The inhibitory effect of ruthenium red on cellular Ca^{2+} uptake also remained after the washing step (see also fig.3). Determinations in the presence of the membrane-impermeant and -permeant heavy metal chelators DTPA and TPEN were also carried out [23,24] and no appreciable variations of the free Ca^{2+} concentration were found.

Fig.4 shows that ruthenium red causes an initial increase followed by a slow decrease of the cytosolic Ca^{2+} concentration of spermatozoa.

4. DISCUSSION

It has been previously reported that ejaculated mammalian spermatozoa are incapable of accumulating exogenous calcium because of the inhibitory effect of a seminal plasma protein [6–8]. We have found that, albeit with a certain variability among different semen samples, Ca^{2+} is always taken up by intact ejaculated bovine spermatozoa. Most likely our isolation procedure causes the partial removal of the Ca^{2+} transport inhibitor [6–8].

The partial inhibition shown by ruthenium red on Ca^{2+} uptake in the presence of 10 μM A23187 is most likely due to the inhibition of the mitochondrial Ca^{2+} uniporter still contributing significantly to the Ca^{2+} transport in the presence of such submaximal amounts of ionophore and in any case during the period elapsing between the ionophore permeabilization of plasma and mitochondrial membranes.

We believe that the discrepancy between our results and those of Singh et al. [4], who found no inhibition by ruthenium red on spermatozoal Ca^{2+} uptake, is probably due to the fact that they used epididymal spermatozoa of guinea pig instead of ejaculated bovine spermatozoa.

During the last decade ruthenium red has been widely used to inhibit specifically the uptake of Ca^{2+} by isolated mitochondria. It has also been found that ruthenium red inhibited calcium uptake in cells such as intact Ehrlich ascites tumor cells [29] or rat hepatocytes [30] and prevented the calcium-induced increase of phosphate uptake in bovine spermatozoa [15]. Recently, McCormack

and England [31] found that ruthenium red inhibited the activation of pyruvate dehydrogenase caused by positive inotropic agents in perfused rat heart. However, the question as to whether ruthenium red acts at the outer cell surface or penetrates into the cytoplasm remains open.

Here we have presented evidence that ruthenium red, added to suspensions of intact spermatozoa, is capable of blocking the mitochondrial Ca^{2+} uniporter in situ. This causes an inhibition of cellular calcium uptake and cycling supporting the evidence, also obtained with the mitochondrial respiration inhibitor and with the uncoupler, that Ca^{2+} uptake by mitochondria accounts almost entirely for the Ca^{2+} accumulation by the whole cells.

It has been recently reported that commercial ruthenium red is a non-homogeneous mixture of different compounds [32]. Most probably, only a fraction of the commonly used ruthenium red enters into the cells and blocks the mitochondrial Ca^{2+} uptake. As a consequence, a mitochondrial Ca^{2+} efflux, via the ruthenium red-insensitive pathway [33], occurs with a resulting increase of the cytosolic free Ca^{2+} concentration. This suggests that, under our conditions, the rate of mitochondrial ruthenium red-insensitive efflux is higher than the rates of Ca^{2+}-extruding systems of the cellular membrane.

The increase of cytosolic free Ca^{2+} might accelerate the acrosomal reaction [9–11]; indeed, preliminary experiments indicate that ruthenium red induces capacitation of mammalian spermatozoa.

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