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Is Zn^{2+} transported by the mitochondrial calcium uniporter?

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Abstract Zinc ions were found to inhibit Ca^{2+} uptake by rat liver mitochondria driven by succinate respiration but not that by a valinomycin-induced membrane potential. Zn^{2+} at 1 μM or higher concentrations induced a lowering of the membrane potential under the former but not the latter conditions. It is concluded that it is the lowered membrane potential in the presence of Zn^{2+} that reduces the rate of respiration-driven Ca^{2+}. Ruthenium red was found to inhibit the uptake of Zn^{2+} but had no influence on its action upon the membrane potential. Zn^{2+} did not affect the Ruthenium red-insensitive Ca^{2+} efflux. Ca^{2+} stimulated the uptake of Zn^{2+}. It is concluded that Zn^{2+} may be transported by the mitochondrial calcium uniporter but that it may have access to sites required for inhibition of respiration by other routes.

Key words: Calcium; Calcium uniporter; Cyclosporin A; Liver mitochondria; Membrane potential; Mitochondrial membrane permeabilisation

1. Introduction

When administered in moderate amounts to rats in vivo, Zn^{2+} is accumulated mainly in the liver mitochondria where succinate dehydrogenase activity is increased [1]. Zn^{2+} was also found to stimulate the activity of cytochrome oxidase and to increase the level of ATP in the cytosol of hepatocytes [2]. Zn^{2+} has, on the other hand, been shown to be highly toxic to cell cultures, and is taken up by the cells by Ca^{2+} channels or, when bound to transferrin, via the transferrin receptor [3]. In cells and in vitro Zn^{2+} inhibits mitochondrial respiration [4] at different points in Complex III of the respiratory chain depending on the amount of Zn^{2+} [4–7]. In rat liver mitochondria there is a high-affinity binding site for Zn^{2+} with a K_D of 1 μM when respiratory chain components are reduced [6]. Like other SH-group reagents, Zn^{2+} promotes energy-dependent uptake of Mg^{2+} [8,9] and K^+ in heart mitochondria if they are present in the medium, and induces mitochondrial swelling [9,10]. Zn^{2+} also promotes binding of the inhibitory subunit IF_1 to the ATP synthase (EC 3.6.1.34) without causing inhibition of the enzyme [11].

Little is known about the mechanism of transport of Zn^{2+} into mitochondria. Zn^{2+} uptake has been reported for rat liver mitochondria [1]. There were no specific inhibitors known for mitochondria Ca^{2+} transport when the early studies cited above were carried out. Zn^{2+} was reported to inhibit the uptake of Ca^{2+} by frog skeletal muscle mitochondria [12]. Rouslin [11] found Ruthenium red not to influence the effect of Zn^{2+} on ATP synthase and concluded that it was not transported on the mitochondrial calcium uniporter. Indeed, heavy metal cations are frequently taken up by mitochondria in a non-specific way [13]. The uniporter has a rather low cation specificity (see review in [13]) determined largely by the ionic radius of the bi- or trivalent cation. Thus, the related Cd^{2+} with an ionic radius of 0.97 Å, close to that of Ca^{2+} (0.99 Å) [14], is transported by the uniporter [15]. Zn^{2+}, however, has an ionic radius of 0.74 Å that is closer to that of Mg^{2+} (0.66 Å) which is not transported. In this study Ruthenium red was found to inhibit the energy- dependent uptake of Zn^{2+} by mitochondria under appropriate conditions, which indicates that it may be transported by the calcium uniporter.

2. Materials and methods

Rat liver mitochondria were prepared by a conventional differential centrifugation method as described elsewhere [16], their protein content was measured by the Lowry procedure [17] with bovine serum albumin as a standard, and respiration was followed polarographically. Changes in $[Zn^{2+}]$ were followed with the metallochromic indicator antipyrylazo III (Fluka AG, Buchs, Switzerland; concentration, 50 μM) employing the wavelength couple 675–690 nm [18]. Ca^{2+} transport was followed by the same technique or with the aid of ^{45}Ca [19]. Safranin at a concentration of 50 μM was used as a probe of the membrane potential [20] with the wavelength pair 554–524 nm. Incubations were carried out at room temperature (22–23°C) in a medium containing 0.25 M sucrose, 2 mM $MgCl_2$, 1 mM KH_2PO_4, 4 mM Tris-succinate and 6 μM rotenone, the pH being adjusted to 7.4 with Tris-base, and suspension density being 0.7 mg protein/ml, if not otherwise mentioned in the legends.

3. Results and discussion

3.1. Zn^{2+} inhibits Ca^{2+} uptake by lowering the membrane potential

Fig. 1 shows the potent inhibition by Zn^{2+} of the uptake of Ca^{2+} by mitochondria respiring on succinate. This confirms earlier reports [12]. This effect may be due to an effect on the calcium uniporter or to an effect of Zn^{2+} on the transmembrane potential that drives the uptake of Ca^{2+} on the uniporter. The inhibitory effect of Zn^{2+} on Ca^{2+} uptake was smaller in the presence of low amounts of Ca^{2+}, which rather points to an effect upon the membrane potential. Indeed, Zn^{2+} decreased the apparent membrane potential when the mitochondria were respiring on succinate (Fig. 2A). In contrast, Zn^{2+} had little, if any, effect on the valinomycin-induced generation of membrane potential or its decay (Fig. 2B). This indicates that the inhibition of net Ca^{2+} uptake by Zn^{2+} is not due to stimulated efflux or induction of a permeability transition but could be due

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Abbreviations: TMPD, *N,N,N,N*-tetramethyl-1,4-phenylenediaminedihydrochloride; Tris, tris-hydroxymethylaminomethane.

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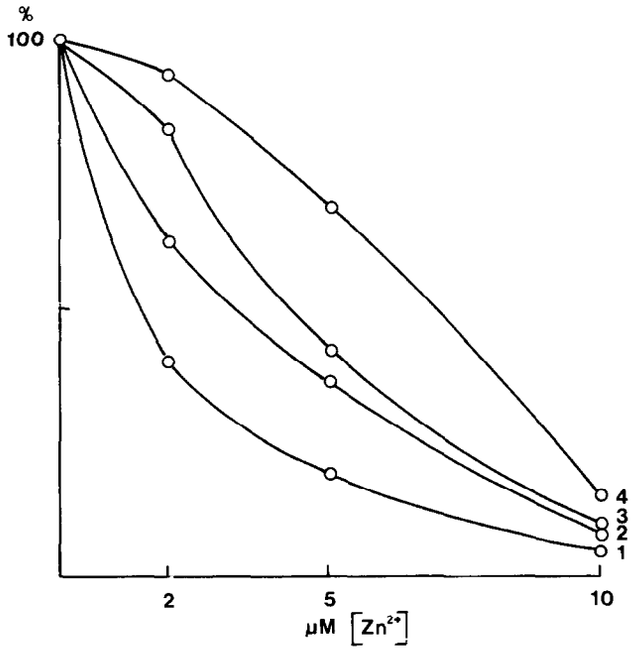

Fig. 1. Inhibition of Ca^{2+} uptake by Zn^{2+}. Uptake was measured by the radioisotope method with sampling at 15 s. The rate of the control without Zn^{2+} is taken as 100%. Ca^{2+} concentrations, (1) 80 μM; (2) 40 μM; (3) 20 μM and (4) 10 μM.

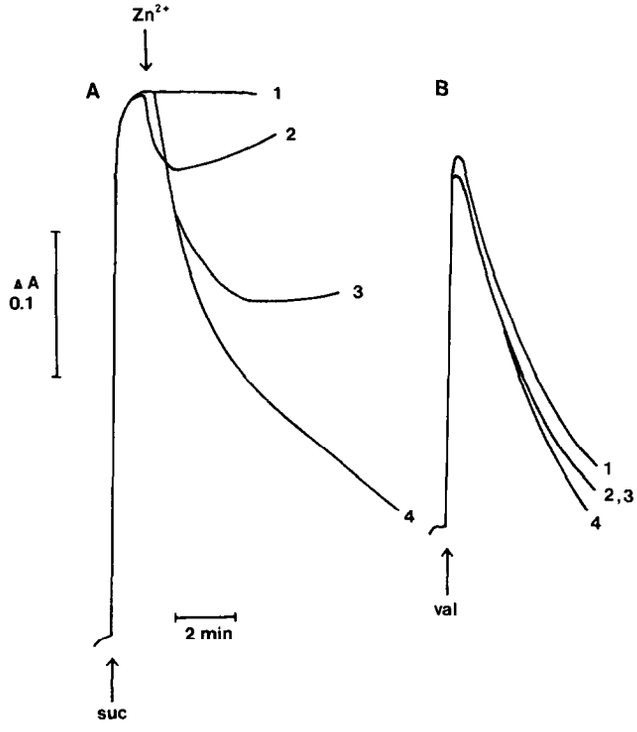

Fig. 2. Effect of Zn^{2+} on the membrane potential. Decrease in membrane potential causes a downward deflection. In A the experimental conditions correspond to those in Fig. 1. In B membrane potential was induced by addition of 0.4 μM valinomycin in a medium in which K^+ was replaced by Na^+ and succinate omitted. The amount of Zn^{2+} was, (1) 0; (2) 1 μM; (3) 4 μM and (4) 10 μM. Mitochondria, 0.5 mg protein/ml.

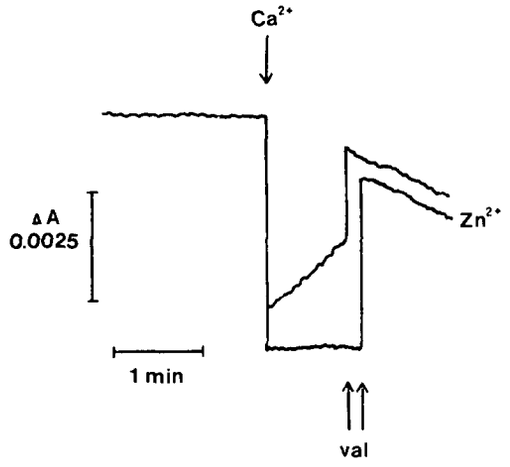

Fig. 3. Zn^{2+} does not inhibit Ca^{2+} uptake driven by the membrane potential. The experimental conditions correspond to those in Fig. 2B. Addition of 20 μM Ca^{2+} was followed by its slow uptake that was inhibited in the presence of 6 μM Zn^{2+}. Addition of valinomycin caused fast uptake of the Ca^{2+}.

to the inhibition of respiration and generation of membrane potential. Thus, when Ca^{2+} uptake was driven by valinomycin-induced membrane potential, added Ca^{2+} was readily taken up also in the presence of Zn^{2+} which, however, inhibited the uptake prior to the addition of valinomycin, i.e. that driven by respiration on endogenous substrates (Fig. 3). The Ruthenium red-insensitive efflux rate of Ca^{2+} was not influenced by Zn^{2+} but was inhibited in the presence of cyclosporin A (Fig. 4). This confirms the finding of Fig. 2B that Zn^{2+} does not cause a permeability transition.

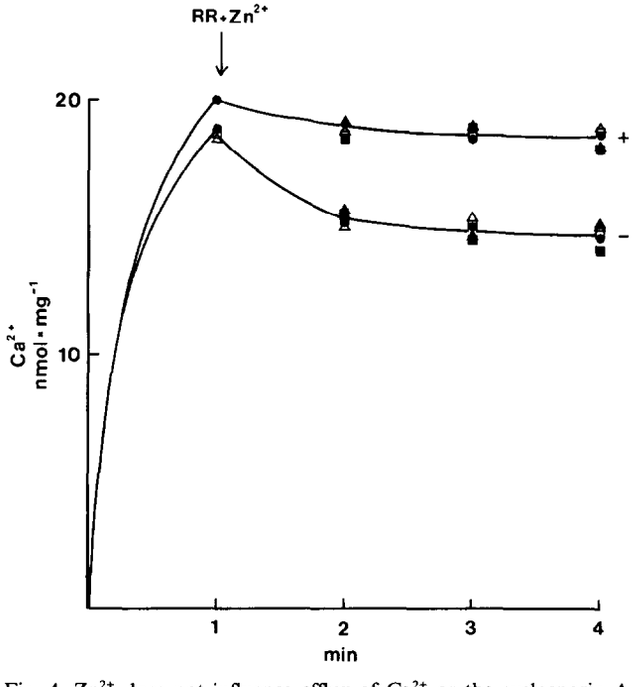

Fig. 4. Zn^{2+} does not influence efflux of Ca^{2+} or the cyclosporin A-sensitive pore. The concentration of Mg^{2+} was 5 mM, and 5 mM Tris-phosphate was present. The different marks correspond to 0, 1, 2, 4 and 10 μM Zn^{2+}. At the arrow Ruthenium red (RR) and Zn^{2+} were added. In the upper trace (+) 0.5 ng/ml cyclosporin A was present.

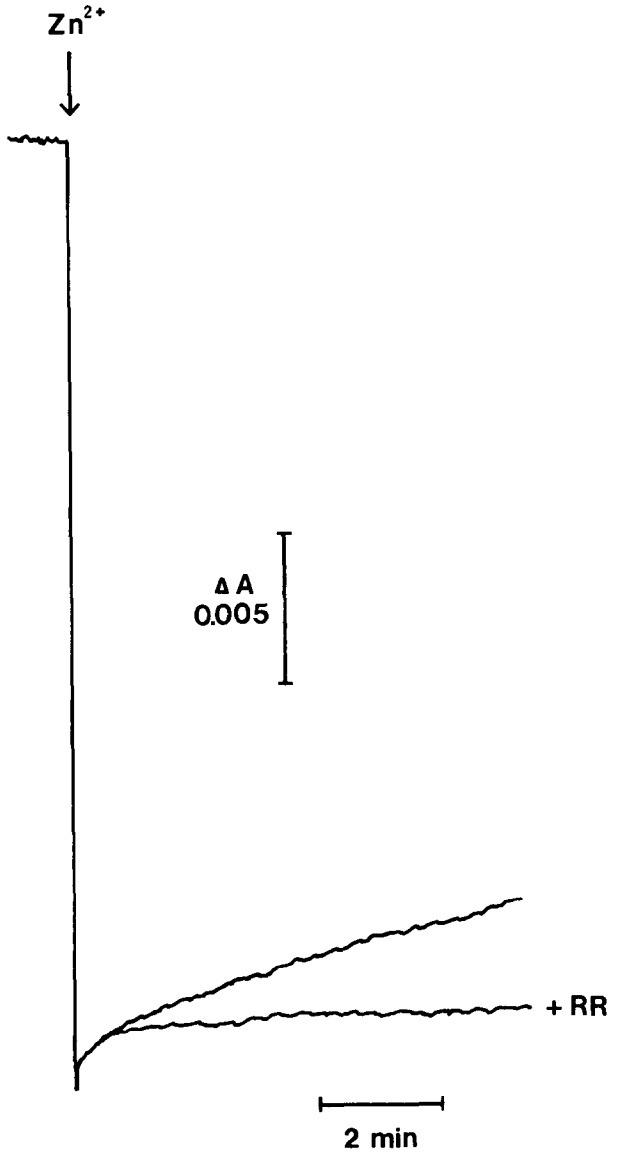

Fig. 5. Inhibition of Zn^{2+} uptake by Ruthenium red. The rat liver mitochondria were respiring on succinate. Addition of 20 μM Zn^{2+} caused a downward deflection followed by a slow upward deflection of the trace, indicating uptake of the cation. This was prevented by Ruthenium red, RR.

3.2. *Inhibition of Zn^{2+} uptake by Ruthenium red*

Fig. 5 shows a trace of Zn^{2+} uptake and its inhibition by Ruthenium red. In a control experiment, the uptake of Ca^{2+} was followed by the radioisotope method using ^{45}Ca. No changes in Ca^{2+} were found after the addition of Zn^{2+} (not shown). This shows that the upper trace indeed represents an uptake of Zn^{2+}, and not Ca^{2+}, on the uniporter.

From these findings it could be expected that the effect of Zn^{2+} on the membrane potential generated by succinate respiration would be prevented by Ruthenium red. However, Ruthenium red did not prevent the drop in membrane potential caused by addition of 1 or 10 μM Zn^{2+} (data not shown). This was true also when Mg^{2+} was omitted and K^+ replaced by Na^+, i.e. in the absence of cations the transport of which is stimulated by Zn^{2+}. The situation is analogous to the finding of Rouslin [11] in that Ruthenium red did not prevent the effects of Zn^{2+} on the binding of the inhibitory subunit to ATP synthase. Either the Zn^{2+}-binding sites involved in these effects are accessible to Zn^{2+} from the cytosolic side or there are other transport mechanisms for Zn^{2+} than the calcium uniporter. Considering the closeness of the ionic radii of Mg^{2+} and Zn^{2+} it is conceivable that these two cations could be transported by the same mechanism. The trace of Zn^{2+} uptake in the presence of Ruthenium red in Fig. 5 does not exclude uptake of some Zn^{2+} immediately following its addition but would not differentiate this from a binding to external sites. The experiment thus does not support a significant transport of Zn^{2+} by other pathways.

3.3. *Modulation of Zn^{2+} uptake by Ca^{2+}*

Fig. 6 shows experiments in which varying amounts of Zn^{2+} followed by 40 μM Ca^{2+} were added to mitochondria respiring on TMPD and ascorbate. The uptake of at least the higher amounts of Zn^{2+} had not yet reached a steady state before the addition of Ca^{2+}. Ca^{2+} was quickly taken up by the mitochondria but the traces continued above the level existing prior to the addition of Ca^{2+}. This indicates stimulation by Ca^{2+} of the uptake of Zn^{2+}. In a control experiment using the highest amounts of Zn^{2+}, Ruthenium red was added simultaneously with Ca^{2+} (trace 6). Comparing this trace with the corresponding trace without the inhibitor (trace 5) reveals that the stimulated uptake is mainly due to the uniporter being sensitive to Ruthenium red. In the control experiment (trace 1) without addition of Zn^{2+}, there is only a slight overshoot. These data show that Ca^{2+} may greatly enhance the uptake of Zn^{2+} on the calcium uniporter. This is analogous to the stimulation of the uptake of Ca^{2+} by Ca^{2+} itself [21] that may lead to an overshoot in the uptake [22].

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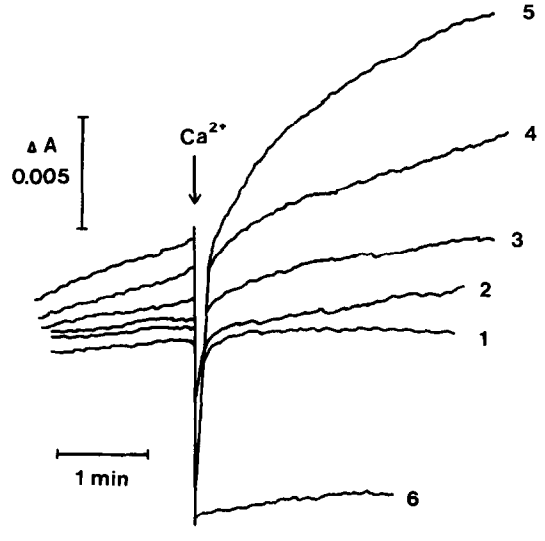

Fig. 6. Ca^{2+} stimulates the uptake of Zn^{2+} driven by respiration of TMPD + ascorbate. Varying amounts of Zn^{2+} were present and had partly been taken up: (trace 1) 0 μM; (2) 5 μM; (3) 10 μM; (4) 20 μM; (5) 40 μM, and (6) 20 μM Zn^{2+} and Ruthenium red. The addition of Ca^{2+} was at 40 μM. The concentration of ascorbate was 2 mM and of TMPD 0.4 mM.

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