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The pH-dependent anion-conducting channel of the mitochondrial inner membrane is potently inhibited by zinc ions

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Zinc is a potent reversible inhibitor of the pH-dependent anion-conducting channel in the mitochondrial inner membrane, 50% inhibition was produced by 1.5 μM added Zn^{2+} at which point free Zn^{2+} was $\leq 10^{-8}$ M. Inhibition by Zn^{2+} is rapid but can be prevented or rapidly reversed by excess EDTA. Concentrations of Zn^{2+} higher than 4 μM caused reversal of inhibition to a variable extent depending on the anion. Under these conditions Zn^{2+} did not inhibit ribose entry, the phosphate transporter, or the pH-insensitive component of the NO_3^- uniport.

Anion channel, Anion uniport; Mitochondrion; Mitochondrial inner membrane; Zinc; Inhibition

1. INTRODUCTION

Zinc ions block chloride and anion channels in the plasmalemma with IC_{50} values around 0.1 mM [1–5] and at 0.1–1.0 mM have been used to block proton channels in snail neurones [6,7]. The Ca^{2+}-sensitive pore forming activity of cytolysin is also inhibited by Zn^2 ions [8].

Zn^{2+} ions inhibit the mitochondrial electron transfer chain in the cytochrome bc_1 region with an IC_{50} of about 3 μM [9,10] and Lorusso et al. [11] found that Zn^{2+} binds to the core proteins I and II, cytochrome c_1, the Fe-S protein and the 9.2 kDa subunit of this complex.

Brierley's group reported that Zn^{2+} is taken up by mitochondria in an energy-linked process [12] and appears to increase passive permeability to K^+ [13]. Zn^{2+} at 40 μM caused release of Ca^{2+} from mitochondria [14] possibly by inhibition of respiration rather than a direct effect on a Ca^{2+}-transporter, and Zn^{2+} at > 12.5 μM reversed inhibition of the F_1 ATPase of sub-mitochondrial particles by the natural inhibitor protein [15].

In view of these effects of Zn^{2+} and the modulation of anion transport via the mitochondrial inner membrane pH-dependent anion conducting channel by divalent cations (activation by Ca^{2+} and inhibition by Mg^{2+} [16–20] we investigated and report here the effects of zinc ions on this channel.

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Abbreviations: IC_{50}, concentration producing 50% inhibition; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone.

2. EXPERIMENTAL

HEPES, TES, Rotenone and Antimycin A were obtained from Sigma. FCCP and ammonium isethionate from Aldrich, and ammonium chloride and zinc chloride were analytical grade from Merck. Ethanesulphonic acid and fumaric acid were obtained from Fluka, neutralised and adjusted to pH 8.0 with ammonium hydroxide solution.

Mitochondria were prepared from the livers of adult Wistar rats as described previously [16,21] with 0.5 mM EGTA in the homogenisation and first wash media.

The anion uniport was assayed by light scattering changes produced by passive osmotic swelling of mitochondria suspended in ammonium salt media in the presence of rotenone, antimycin A and FCCP with controls in the absence of FCCP as described previously [16]. For some experiments, mitochondria were depleted of Ca^{2+} and Mg^{2+} by the method of Beavis and Garlid and assayed in KCl media [17].

True initial rates cannot be estimated because during the first few seconds the mitochondria adjust to the change of medium, but we have estimated rates in the period 8–20 s after adding mitochondria when the swelling has settled to an approximately linear rate, Fig. 1 trace (a) or, in the presence of zinc, Fig. 1 trace c, there is an inflexion point or linear region. This approximation to initial rates minimises problems produced as time and swelling proceed: notably release of Ca^{2+} ions, lysis of some mitochondria and any time-dependent redistribution of the zinc to other binding sites or uptake and sequestration of zinc in the mitochondrial matrix.

In view of these approximations and the time-dependence of inhibition by zinc, we have characterised the action of zinc with a purely empirical IC_{50}.

Zinc was measured by atomic absorption spectrophotometry using a Varian Spectra AA-10 with a GTA-96 Graphite Tube Atomizer.

FigP version 6.0, BIOSOFT, Cambridge, UK was used to draw Figs. 2, 3 and 4 and for non-linear regression.

3. RESULTS

The rate of swelling of mitochondria suspended in isotonic ammonium chloride in the presence of the uncoupler FCCP, which measures the rate of Cl^- ion uniport

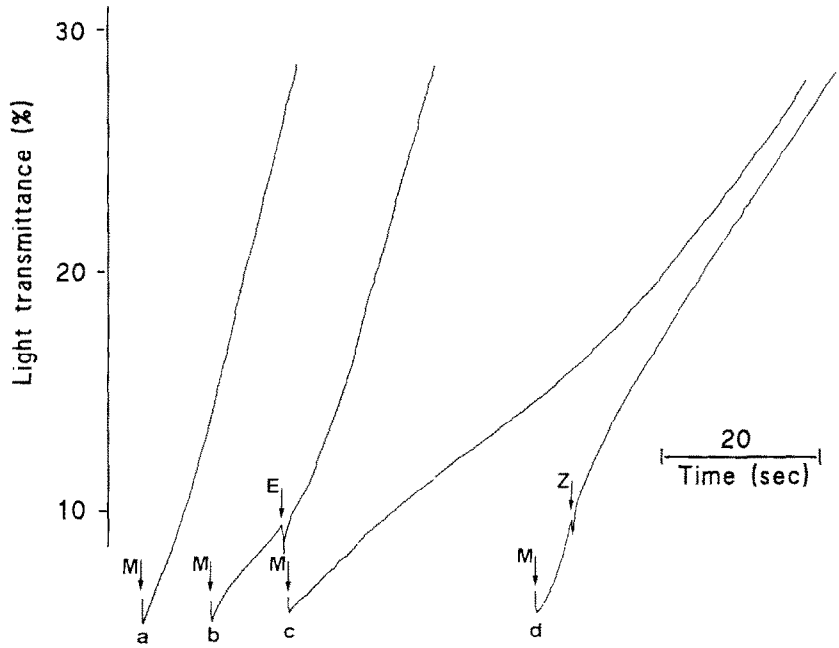

Fig. 1. Recordings of light scattering showing the effect of Zn^{2+} ions on mitochondrial swelling in 100 mM NH_4Cl in the presence of rotenone, antimycin A and FCCP. Conditions were as described in the text. Arrows M show the addition of mitochondria (a) No further additions. (b) 4 μM Zn added prior to mitochondria, 5 μM EDTA added at arrow E. (c) 4 μM Zn added prior to mitochondria. (d) 4 μM Zn added at arrow Z.

via the pH-dependent anion conducting channel, was markedly inhibited by Zn^{2+} ions in the concentration range 1–5 μM, Fig. 1c. Inhibition was prevented by EDTA at concentrations equal to or greater than that of zinc ions and addition of excess EDTA during the process of anion entry produced rapid reversal of inhibition, Fig. 1b. When zinc was added to mitochondria which were already swelling, trace d of Fig. 1, the inhibition is substantial (compare with trace a) but not as great as the maximal inhibition produced when zinc is present initially (trace c). Trace d also shows that inhibition by zinc is time dependent but fairly rapid on the time scale of these experiments with a $t_{1/2}$ of about 2 s (estimated by fitting a first-order exponential expression to the development of inhibition [22] by non-linear regression). This time dependence may lead to an underestimation of inhibition, particularly at low zinc concentrations.

In the absence of added Ca^{2+}, Fig. 2, maximal inhibition, 90% was produced by Zn^{2+} at 4 μM and higher concentrations of Zn^{2+} partially reversed inhibition. In the absence of added Ca^{2+} and Mg^{2+} the IC_{50} was 1.5 ± 0.4 μM (mean ± S.E. for 8 estimations). As shown in Fig. 2, the IC_{50} was not changed by the addition of 1.5 mM Mg^{2+} ions; 10 μM Ca^{2+} which greatly increases the rate in the absence of Zn^{2+} appears to produce a small increase in the IC_{50} for Zn^{2+}. Increasing the Ca^{2+} concentration to 30 μM produced no further increase in the IC_{50}. The rate at maximal inhibition by Zn^{2+} was not greatly affected by the presence of Mg^{2+} or Ca^{2+} but because the rate in the absence of Zn^{2+} was increased by Ca^{2+} and decreased by Mg^{2+}, maximal inhibition by Zn^{2+} was about 97% in the presence of Ca^{2+} but only 60% in the presence of Mg^{2+}.

The entry of other anions via the pH-dependent anion-conducting channel was also inhibited by Zn^{2+}, Fig. 3. With isethionate and ethanesulphonate (not

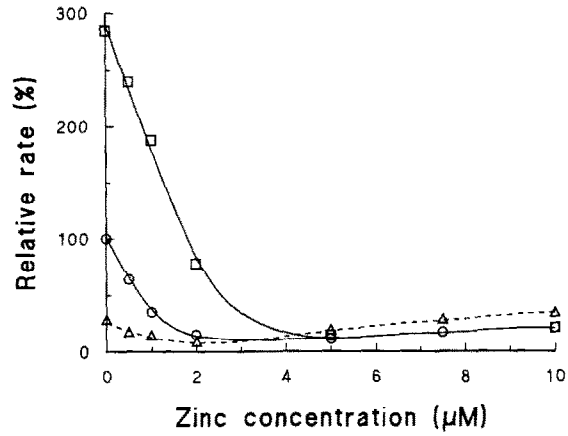

Fig. 2 Effect of Zn^{2+} on the rate of chloride uniport in the presence and absence of Ca^{2+} and Mg^{2+} ions. Chloride uniport was measured by light scattering changes of mitochondria suspended in NH_4Cl medium as described in section 2 and is expressed as a percentage of the rate in the absence of added metal ions. When present, Zn^{2+}, Ca^{2+} and Mg^{2+} were added to the medium prior to addition of mitochondria. ○, No addition other than Zn^{2+} as indicated on abscissa; □, 10 μM Ca^{2+} present, △, 1.5 mM Mg^{2+}.

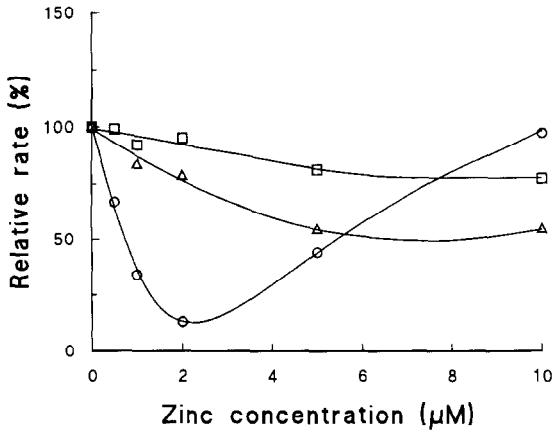

Fig. 3. Effect of Zn^{2+} on uniport of various anions. Experiments were conducted as in Fig. 2(a) except that the media contained the ammonium salts of the following anions adjusted to pH 8.0: ○, 100 mM isethionate; □, 83 mM maleate; △, 83 mM succinate. Rates are expressed as percentages relative to the rate in the absence of Zn^{2+} for each anion

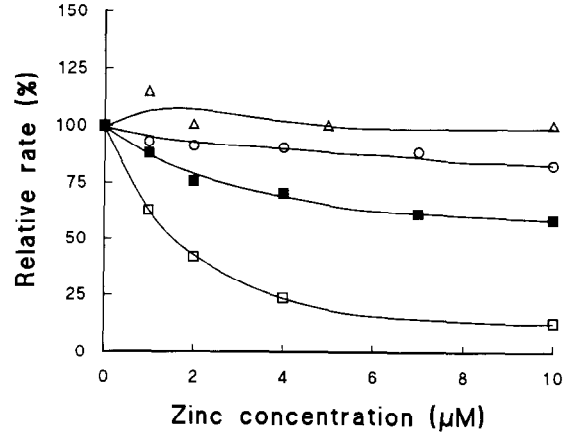

Fig. 4. Effect of Zn^{2+} on mitochondrial swelling in various media. △, 250 mM ribose containing 5 mM HEPES KOH, pH 8.0, no. FCCP; ○, 83 mM ammonium phosphate adjusted to pH 7.4 with ammonium hydroxide solution, no FCCP; ■, 100 mM NH_4NO_3, pH 7.0, 7.5 μM FCCP; □, 100 mM NH_4NO_3, pH 8.0, 7.5 μM FCCP. Rates are expressed as percentages relative to the rate in the absence of Zn^{2+} for each medium.

shown) the effective Zn^{2+} concentration was similar to that in the chloride assay but with maleate, succinate and fumarate (not shown) the effective concentration of Zn^{2+} was shifted to a higher range. This can be explained by chelation of Zn^{2+} by these anions: published K_1 values for succinate (50 M^{-1}) and maleate (100 M^{-1}) [23] show that the free Zn^{2+} ion concentration in these media would be decreased by factors of approximately 5 and 9, respectively. With isethionate and ethanesulphonate the reversal of inhibition by high Zn^{2+} concentrations was much greater than with Cl^-. Thus unlike the inhibition of anion entry the reversal of inhibition is anion-specific.

Entry of ribose was not inhibited by Zn^{2+} nor is phosphate entry via the phosphate transporter, Fig. 4. Entry of nitrate at pH 7 was only partially inhibited by Zn^{2+} but at pH 8 nitrate entry was inhibited to a much greater extent. This is consistent with the ability of nitrate to cross the membrane by non-specific diffusion, apparently a Zn^{2+}-insensitive process, which forms a large proportion of the total nitrate uniport at pH 7 but only a small proportion at pH 8.

In 0.1 M NH_4Cl Zn^{2+} ions are complexed with NH_3, mostly as the $Zn(NH_3)_4$ complex and this might be the inhibitory species. However, the potent inhibition of Cl^- entry by Zn^{2+} in a KCl medium, Fig. 5, shows that formation of an NH_3 complex is not required for inhibition.

Much of the Zn^{2+} added to the assay medium is bound to the mitochondria and therefore the concentration of free Zn^{2+} was estimated by centrifuging the suspension at 12,000 × *g* for 5 min and measuring the zinc concentration in the supernatant fluid. At 2 μM total added zinc the concentration in the supernatant was approximately 10^{-8} M.

4. DISCUSSION

We conclude from the lack of effect on other transport processes that the inhibition of anion entry via the pH dependent anion conducting channel in the mitochondrial membrane by Zn^{2+} ions is a specific effect on this channel and not a general perturbation of membrane function. The inhibition is rapid in onset and readily reversible by chelation of free Zn^{2+} ions with EDTA. The finding that Cl^- which is transported rapidly and isethionate and ethanesulphonate which are transported slowly are affected to a similar extent and at similar Zn^{2+} concentrations indicates that inhibition is produced by an interaction between Zn^{2+} ions and channel components. The reversal of inhibition at higher Zn^{2+} concentrations differs in extent with different anions. This reversal cannot be explained by formation of soluble zinc–anion complexes which, as with the dicarboxylates, would increase the concentration of zinc required for both inhibition and reversal and possibly also limit the maximal inhibition. Nor can it be explained by formation of insoluble salts (not detected either visually or by light scattering prior to addition of mitochondria) which would limit the maximal concentration of free zinc and thereby either limit the maximal inhibition or the extent of reversal but would not produce reversal of inhibition with increasing total zinc concentration. It seems probable that reversal involves a ternary interaction between channel components, Zn^{2+} ions and anions.

Although the IC_{50} for total Zn added was 1.5 μM the concentration of zinc in the supernatant medium was about 10^{-8} M. Because zinc may be bound to fragments of mitochondria and complexed to soluble components

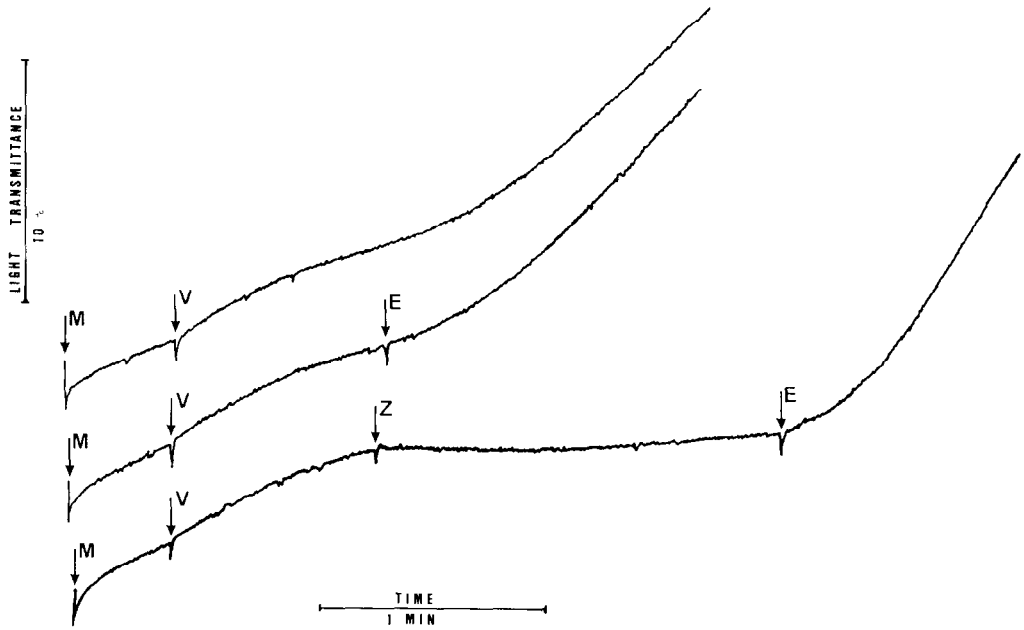

Fig. 5. Effect of Zn^{2+} on mitochondrial swelling in KCl medium. Conditions and measurements were as in Fig. 1 except that the medium was 55 mM KCl, 5 mM TES-KOH, pH 7.5, and the mitochondria had been depleted of Ca^{2+} and Mg^{2+} by the method of Beavis and Garlid [17]. Arrows show additions as follows: M, mitochondria 0.4 mg protein; V, valinomycin at 1 nmol/mg mitochondrial protein; E, 10 μM EDTA; Z, 2 μM Zn^{2+}.

such as adenine nucleotides this is considered to be a maximal value for the K_D for inhibition of the anion conducting channel. Even so it is much higher than Williams [24] estimate of 10^{-12} M for the concentration of free Zn^{2+} in the cytosol or Simons [25] value of 2.4×10^{-11} M for free Zn^{2+} in human red blood cells and it is therefore unlikely, but not impossible, that Zn^{2+} modulates anion movement through the pH-dependent anion-conducting channel in vivo.

A high conductance channel in the inner mitochondrial membrane detected by patch clamping techniques was not affected by 0.1 mM $ZnCl_2$ [26] which may indicate that it is a different channel, or that the channel has lost Zn^{2+}-sensitivity during preparation for patch clamping, or that at this Zn^{2+} concentration the inhibitory effect has been reversed. It is also possible that the Zn^{2+}-sensitive channel scavenged Zn^{2+} during preparation for patch clamping, was therefore already blocked and not detected.

Our observations show that the pH-dependent mitochondrial inner membrane anion-conducting channel is much more sensitive to inhibition by Zn^{2+} than the plasmalemma channels [1–7] and many of the other effects of Zn^{2+} on mitochondria [13–15]. We have confirmed, data not shown, that inhibition of the cytochrome bc_1 [9–11] complex requires Zn^{2+} concentrations which are slightly higher (IC_{50} 4 μM) than those which inhibit anion uniport.

As well as revealing the need to exercise care to prevent inhibition by adventitious Zn^{2+} binding, this potent inhibition by Zn^{2+} should be a useful tool for investigation of the pH dependent anion-conducting channel of the mitochondrial inner membrane.

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