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A comparison of Zn^{2+} - and Ca^{2+} - triggered depolarization of liver mitochondria reveals no evidence of Zn^{2+} -induced permeability transition

Michael J. Devinney¹, Latha M. Malaiyandi², Olga Vergun³, Donald B. DeFranco^{1,4}, Teresa G. Hastings^{3,4}, and Kirk E. Dineley²

¹Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261

²Department of Biology, Francis Marion University, Florence SC 29501

³Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh PA 15261

⁴Center for Neuroscience, University of Pittsburgh, Pittsburgh, PA 15261

Abstract

Intracellular Zn^{2+} toxicity is associated with mitochondrial dysfunction. Zn^{2+} depolarizes mitochondria in assays using isolated organelles as well as cultured cells. Some reports suggest that Zn^{2+} -induced depolarization results from the opening of the mitochondrial permeability transition pore (mPTP). For a more detailed analysis of this relationship, we compared Zn^{2+} -induced depolarization with the effects of Ca^{2+} in single isolated rat liver mitochondria monitored with the potentiometric probe Rhodamine123. Consistent with previous work, we found that relatively low levels of Ca^{2+} caused rapid, complete and irreversible loss of mitochondrial membrane potential, an effect that was diminished by classic inhibitors of mPT, including high Mg^{2+} , ADP and cyclosporine A. Zn^{2+} also depolarized mitochondria, but only at relatively high concentrations. Furthermore Zn^{2+} -induced depolarization was slower, partial and sometimes reversible, and was not affected by inhibitors of mPT. We also compared the effects of Ca^{2+} and Zn^{2+} in a calcein-retention assay. Consistent with the well-documented ability of Ca^{2+} to induce mPT, we found that it caused rapid and substantial loss of matrix calcein. In contrast, calcein remained in Zn^{2+} -treated mitochondria. Considered together, our results suggest that Ca^{2+} and Zn^{2+} depolarize mitochondria by considerably different mechanisms, that opening of the mPTP is not a direct consequence of Zn^{2+} -induced depolarization, and that Zn^{2+} is not a particularly potent mitochondrial inhibitor.

Keywords

Intracellular zinc; intracellular calcium; reactive oxygen species; rhodamine123; mitochondrial transmembrane potential; cyclosporine A

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Corresponding author: Kirk E. Dineley, PhD, 201A McNair Science Building, Francis Marion University Florence, SC 29501, Phone +1 843-661-1413, fax +1 843-661-4660, kdineley@fmarion.edu.

*Conflict of Interest Statement

The authors have no conflict of interests to declare.

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Introduction

Cytotoxicity resulting from excessive intracellular free zinc ($[Zn^{2+}]_i$) may be an important contributor to numerous pathologies, including diseases such as Parkinson's, Alzheimer's, and stroke [1]. Experiments in cultured cells showed that high $[Zn^{2+}]_i$ is frankly toxic, and that oxidant-induced cell injury relies at least partially on mobilization of intracellular zinc stores [2, 3]. Precisely how Zn^{2+} kills cells remains uncertain, but there is evidence that high Zn^{2+} perturbs enzyme activity, results in oxidative stress, and impairs cellular energy production [for reviews see 4, 5, 6]. In fact Zn^{2+} was identified as a possible inhibitor of mitochondria shortly after mitochondria themselves could be studied properly [7]. But despite a long if uneven history of investigation, it remains unclear exactly how Zn^{2+} affects mitochondria, and if those effects are importantly related to Zn^{2+} toxicity in living cells. The mechanism of Zn^{2+} -induced inhibition of mitochondria has been suggested to involve inhibition of complexes of the electron transport chain or inhibition of matrix enzymes [8, 9]. Other studies suggested that Zn^{2+} is similar to Ca^{2+} in its ability to open the mitochondrial permeability transition pore (mPTP) [10, 11].

mPT involves the opening of a large, non-specific pore on the mitochondrial membranes that results in mitochondrial uncoupling, loss of the mitochondrial transmembrane electrochemical gradient ($\Delta\psi_m$), efflux of matrix solutes and, at extreme, fragmentation of the mitochondrial membranes [reviewed by 12, 13, 14]. While the molecular composition of the pore remains undetermined, its function has been fairly well-characterized. Thus the consequences of mPTP opening include collapse of mitochondrial energy production and the release of apoptogens into the cytosol, such as cytochrome *c* and apoptosis-inducing factor (AIF). Conditions favoring mPT include decreased ATP/ADP ratio, increase in inorganic phosphate, low $\Delta\psi_m$, elevated ROS, and high Ca^{2+} . Because Ca^{2+} -triggered mPT requires mitochondrial Ca^{2+} overload, agents that inhibit Ca^{2+} import such as ruthenium red and Ru360 prevent Ca^{2+} -induced mPT. Other agents that inhibit pore opening include Mg^{2+} , adenine nucleotides and cyclosporine A.

In the present study we used a model of single mitochondria isolated from rat liver and visualized by fluorescence microscopy to further clarify the effect of Zn^{2+} on mitochondria in general, and to specifically examine the ability of Zn^{2+} to initiate mPT. By comparing the effect of Zn^{2+} with Ca^{2+} , a classic mPT inducer, we found that (i) Ca^{2+} and Zn^{2+} depolarize mitochondria by different mechanisms (ii) Zn^{2+} does not directly initiate mPTP and (iii) Zn^{2+} is not a particularly effective or potent inhibitor of mitochondria. Portions of this work were presented previously in abstract form [15].

Materials & Methods

Materials

Rhodamine 123 (Rh123), tetramethylrhodamine methyl ester (TMRM) and calcein-AM were purchased from Invitrogen (Carlsbad, CA). All other chemicals and reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Isolation of rat liver mitochondria

All procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Liver mitochondria were isolated from adult Sprague-Dawley rats as previously described by Vergun and Reynolds [16]. Isolation buffer contained (in mM): 225 mannitol, 75 sucrose, 0.5 EDTA, 5 HEPES and 1mg/mL fatty acid free BSA, with pH adjusted to 7.4 with KOH. All isolation

procedures were carried out at 0–2 °C. Briefly, liver tissue was homogenized using a glass/glass homogenizer, centrifuged for 10 min at 1300g, the pellet was discarded, and the supernatant was centrifuged again for 10 min at 10,000g. The resulting supernatant was removed together with the top layer of the pellet, which contains damaged mitochondria, and the dark-brown “dense” mitochondrial pellet was resuspended in isolation buffer and centrifuged again for 10 min at 10,000g. For the final spin (11,000g for 10 min) the pellet was resuspended in the isolation buffer where BSA was omitted and the concentration of EDTA was reduced to 0.1 mM. The final mitochondrial pellet obtained was then gently resuspended in the isolation buffer and stored on ice prior to experiments. Mitochondria prepared this way are viable for 6 to 8 hours after isolation, as determined by their ability to maintain a transmembrane potential in the presence of oxidizable substrates [17].

Fluorescence microscopy

All imaging experiments were performed at room temperature in KCl-based buffer containing (in mM): 125 KCl, 2 K₂HPO₄, 5 HEPES, 1 MgCl₂, 5 malate, and 5 glutamate; pH was adjusted to 7.0 with KOH. The buffer was pre-treated in Teflon-coated Nalgene containers in 2 g/L buffer of Chelex 100 resin for 1 hour prior to use in experiments to remove contaminating metals from the solution. The MgCl₂ was added to the solution after the Chelex treatment to prevent loss of Mg²⁺ due to chelation by the resin. To attach the mitochondria, 31mm glass coverslips were washed with 70% ethanol, rinsed with H₂O, and dried prior to use. Approximately 20 µl of mitochondrial suspension was placed on the coverslip at a concentration of 15–20 mg protein/ml for 20–30 seconds and then gently washed to remove unattached mitochondria. Coverslips were then placed into a 700 µl perfusion chamber and mounted onto a microscope fitted for fluorescence imaging, as described below. The coverslip was superfused with KCl buffer at 7 ml/min. Typically, mitochondria remained attached at a density of 400,000–500,000/mm², which yielded approximately 2000 mitochondria in a field observed with a 100X objective.

For ψ_m measurements we used the potentiometric probe Rh123 at non-quenching concentrations (100 nM), present in the perfusion medium during the experiment (no pre-loading was necessary). Rh123 is a cationic, lipophilic species that accumulates in the matrix of polarized mitochondria. Upon depolarization, Rh123 is lost into the surrounding medium, and therefore a decrease in the fluorescence signal from individual mitochondria reflects membrane depolarization. At the beginning of the experiments about 60–70% of mitochondria attached to the coverslip were polarized. For dye retention experiments, attached mitochondria were incubated with 1 ml of 1 µM calcein-AM in substrate-free KCl buffer containing 200 µM EDTA and 5 mg/ml of fatty acid-free BSA at 37 °C for 10 minutes. The incubation solution was washed off with Chelex-treated, KCl-based buffer (no EDTA) and then mitochondria were briefly perfused with 40 nM TMRM. Only the mitochondria showing bright TMRM fluorescence reflecting a stable ψ_m were pre-selected for analysis. ZnCl₂, CaCl₂, and other reagents were diluted in Chelex-treated KCl buffer from stock solutions. The lack of soluble chelating agent, e.g. EGTA or EDTA, in the buffer allowed for relatively unambiguous determination of free metal concentrations in the superfusate.

For fluorescence recordings, we used a BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 100X water-immersion quartz objective. Excitation light was generated by a 75W xenon lamp-based monochromator (T.I.L.L. Photonics GmbH, Martinsried, Germany), and emitted light was detected using a CCD camera (Orca; Hamamatsu, Shizouka, Japan). Rh123 or calcein was illuminated at 490 nm, and light passed through a 500 nm long pass dichromatic mirror and a 535/25 nm band pass filter (Omega Optical, Brattleboro, VT, USA). TMRM was excited at 550 nm, and light was modified by a 570 nm long-pass dichromatic mirror and a 605/55nm band pass filter.

Fluorescence data was acquired and analyzed using SIMPLE PCI software (Compix Inc., Cranberry, PA). Fluorescence was measured in 40–80 individual mitochondria for each coverslip. Objects smaller than 0.3 μm in diameter were not considered mitochondria and were excluded from analysis. Background fluorescence was averaged from three to four mitochondrion-free regions and subtracted from all the signals. All experiments were repeated four to six times using mitochondria prepared from at least three different animals.

Data Analysis

Our first data analysis (e.g. Figure 2A) was based on the number of mitochondria that were *fully* depolarized by metal treatment. In this case, full depolarization was arbitrarily set to a raw Rh123 fluorescence less than 40 units (about 20% of starting fluorescence). However, because Zn^{2+} usually resulted in only partial depolarization, we also analyzed that data by calculating the percent reduction of Rh123 fluorescence for each mitochondrion (e.g. Figure 2B). Statistical analysis was performed using GraphPad Prism 4.03 (Graph Pad Software, San Diego, CA). All data are presented as mean $\pm$ S.E. Comparisons were made using two-tailed Student's t-test, with p-values of less than 0.05 regarded as significant. For all conditions, experiments were repeated on 3 to 7 coverslips using mitochondria prepared from at least 3 different animals. Typically, 40–80 mitochondria were sampled on each coverslip to generate a mean data set, with N usually referring to the number of coverslips sampled for each condition.

Results

1. The dynamics of Ca^{2+} - and Zn^{2+} -induced mitochondrial depolarization are different

The basic consequences of mPTP opening are mitochondrial uncoupling and a loss of $\Delta\psi_{\text{m}}$. We recorded $\Delta\psi_{\text{m}}$ changes reflected by Rh123 fluorescence in individual mitochondria, where a decrease in the Rh123 fluorescence reflects a decrease in $\Delta\psi_{\text{m}}$. Panels A–H of Figure 1 show concentration responses to Ca^{2+} and Zn^{2+} at 1, 10, 30 and 100 μM . Each trace represents an individual mitochondrion, and each panel is a representative population of mitochondria taken from a single coverslip. Mitochondria were relatively unaffected by a 6-minute treatment with either Ca^{2+} or Zn^{2+} at 1 μM . (It is important to note that during sham wash, i.e. no added metal, about 20% of mitochondria spontaneously depolarized fully; see Figure 2B.) Ca^{2+} at 10 μM caused rapid depolarization that was complete, irreversible, and uniform throughout the entire population (Figure 1B). Increasing Ca^{2+} (30 and 100 μM) only decreased the apparent latency period preceding mass depolarization (Figure 1C and 1D). With Zn^{2+} , the concentration dependence as well as the kinetics of depolarization differed substantially. Zn^{2+} at 10 μM produced partial depolarization that developed slowly but progressed continuously until termination of the Zn^{2+} stimulus, whereupon $\Delta\psi_{\text{m}}$ sometimes recovered partially during washout (Figure 1F). More Zn^{2+} (30 or 100 μM) resulted in greater depolarization that was still relatively slow and reversible (Figure 1G and 1H). Thus Figure 1 reveals important differences between Ca^{2+} and Zn^{2+} effects on mitochondria: Ca^{2+} caused rapid, complete, and irreversible depolarization in an all-or-none fashion. Conversely, Zn^{2+} depolarization was concentration-dependent, slow, and partially reversed by washout.

The summarized data of these experiments are presented in Figure 2. Because of the fundamental differences between Ca^{2+} - and Zn^{2+} -induced depolarization, we used two approaches to quantify the responses (see methods). Figure 2A shows the percentage of mitochondria that depolarized ‘completely’ in response to metal treatment, i.e. the fraction of mitochondria that lost all signal in response to added metal. However, because Zn^{2+} caused only partial depolarization in the majority of the experiments, we also calculated the percentage of depolarization in individual mitochondria for each Zn^{2+} treatment, that is, the

extent of depolarization exhibited by each mitochondrion, averaged for all the mitochondria sampled (Figure 2B). Thus Figure 2A shows that nearly all mitochondria treated with $10 \mu\text{M Ca}^{2+}$ fully depolarized by the end of the treatment, while Zn^{2+} at the same concentrations was no different from sham treatment. However, Figure 2B reveals the gradual concentration response to Zn^{2+} where individual mitochondria increasingly depolarized in response to increasing Zn^{2+} . Figure 2B was also used to approximate an IC_{50} for Zn^{2+} of $18.69 \mu\text{M}$ (13.62 to 30.66 , 95% CI). For Figures 3 and 4, Ca^{2+} data is analyzed by percentage of mitochondria fully depolarized, while Zn^{2+} is quantified by percent depolarization for each mitochondrion.

2. Permeability transition inhibitors protect against Ca^{2+} - but not Zn^{2+} -induced depolarization

To determine the involvement of mPT in the metal-induced mitochondrial depolarization, we used pharmacological modulators of mPT. One of the most widely recognized is cyclosporine A (CsA), an inhibitor of cyclophilin D. CsA ($1 \mu\text{M}$) was added for 2 minutes before and during metal treatments. Compared to Ca^{2+} alone (Figure 3A), CsA resulted in a time delay to depolarization, as well as a decrease in the number of fully depolarized mitochondria at the termination of the Ca^{2+} stimulus. This protection is evident in the sample of mitochondria from a representative traces (Figure 3B), as well as in the summary plots (D and E) of the average percent depolarization over time for each condition. As shown in Figure 3D, approximately 88% of control mitochondria were completely depolarized at the end of $10 \mu\text{M Ca}^{2+}$ while only 36% of CsA-treated mitochondria completely lost $\Delta\psi_m$ by the same time point. Panel 3E shows that CsA protected against higher Ca^{2+} ($30 \mu\text{M}$), though to a lesser extent, depolarizing 100% mitochondria after 3 minutes, whereas 72% depolarized when CsA was included. We tested additional agents known to inhibit Ca^{2+} -induced mPT, including Mg^{2+} (5 mM), and ADP with oligomycin (100 and $2 \mu\text{M}$, respectively). Both treatments inhibited Ca^{2+} -induced depolarization, as indicated by the summary plots presented in Figures 3D and 3E. In summary, these data demonstrate that (i) mPT is readily observable and amenable to manipulation in this model, and that (ii) classic mPT inhibitors can extend the time for a Ca^{2+} stimulus to cause complete depolarization, and reduced the percentage of mitochondria that depolarize completely over the course of the Ca^{2+} exposure.

We also evaluated how mPT inhibitors affected Zn^{2+} -induced depolarization (Figure 4). Mean traces using CsA are presented in Figure 4A and 4B, and summary data for all of the mPT inhibitors (Mg^{2+} , ADP + oligomycin or CsA, each tested at 10 and $30 \mu\text{M Zn}^{2+}$), are presented in Figure 4C. Although all protected to varying extent in the Ca^{2+} experiments, none protected against, or altered the extent or time course of, Zn^{2+} -induced depolarization.

3. Inhibition of the mitochondrial Ca^{2+} -uniporter prevented Ca^{2+} - but not Zn^{2+} -induced depolarization

Ca^{2+} -induced mPT requires an increase in mitochondrial matrix free Ca^{2+} . The route of entry for Ca^{2+} is the mitochondrial Ca^{2+} -uniporter [18]. In isolated brain mitochondria, we previously showed that Zn^{2+} also can enter the mitochondrial matrix via the Ca^{2+} -uniporter [19]. If one assumes that Zn^{2+} depolarizes mitochondria by a mechanism similar to Ca^{2+} , then inhibition of Ca^{2+} -uniporter with ruthenium red should modulate the effect of both metals similarly. As expected, ruthenium red ($2 \mu\text{M}$) greatly reduced Ca^{2+} -induced depolarization (Figure 3C–E). A representative experiment of this series is shown in Figure 3C. However ruthenium red did not change mitochondrial responses to Zn^{2+} (10 and $30 \mu\text{M}$, Figure 4C). This suggests that Zn^{2+} -induced depolarization does not require Zn^{2+} accumulation in the matrix and, more generally, that Ca^{2+} and Zn^{2+} depolarize mitochondria by different mechanisms. This is in broad agreement with our previous work in brain

mitochondria, which showed that inhibition of mitochondrial Zn^{2+} uptake by ruthenium red gave only modest protection against Zn^{2+} -induced depolarization [19].

4. Release of calcein from Ca^{2+} - but not Zn^{2+} -treated mitochondria

To support the data obtained with $\Delta\psi_m$ measurements, we next used a different approach for mPT detection. mPTP opening in cells or isolated mitochondria can be directly visualized by imaging the redistribution of the fluorophore calcein between the mitochondrial matrix and surrounding space [20]. The AM ester of calcein accumulates in mitochondria where, upon de-esterification, it becomes trapped. With a molecular weight of 622, the de-esterified form of calcein is small enough to escape through the mPTP, which accommodates solutes as large as 1.5 kDa (Crompton, 1999). Calcein-loaded mitochondria are visible in the micrographs of Figure 5A. Because calcein was observed in polarized and some partially or completely depolarized mitochondria, we used the potentiometric probe TMRM to preselect only polarized calcein-loaded mitochondria. As shown in Figure 5B, calcein fluorescence remained relatively stable in mitochondria observed over a 10- minute time period. Ca^{2+} (30 μM) caused a rapid and substantial loss of calcein from mitochondria (Figure 5C). In contrast, calcein remained relatively stable in mitochondria treated with Zn^{2+} (30 μM , Figure 5D). Although these experiments press the resolution of light microscopy, close inspection of the digital images suggests that Ca^{2+} caused morphological alteration of mitochondria, while Zn^{2+} left them relatively intact. The fluorescence time courses are summarized in Figure 5E. In this analysis, we considered individual mitochondria that lost 85% of initial signal to have shown 'full dye loss'. Zn^{2+} (30 μM) did not differ from control, while ~80% of mitochondria treated with Ca^{2+} (30 μM) lost their calcein. This data is visual evidence of the inability of Zn^{2+} to induce mPTP.

Discussion

In this study we demonstrated that the inhibitory action of Zn^{2+} on mitochondria differs importantly from that of Ca^{2+} . The Ca^{2+} data assures that in this model mPT is readily observable and can be modulated by classic maneuvers. In contrast to Ca^{2+} , Zn^{2+} depolarization was slow, partial, and reversed with relative ease. While all the mPT inhibitors we used protected against Ca^{2+} , none protected against Zn^{2+} . Mitochondria retained calcein in the face of Zn^{2+} but not Ca^{2+} , further reinforcing the conclusion that Zn^{2+} does not cause mPT. The ruthenium red experiments imply that, in contrast to Ca^{2+} , Zn^{2+} depolarization does not require uniporter uptake. Finally, we must conclude that Zn^{2+} is a relatively weak mitochondrial toxin, given the concentrations necessary to cause even partial depolarization.

A common problem in studies of isolated mitochondria is metal contamination. Excess Ca^{2+} is toxic for obvious reasons, and adventitious transition metals are problematic as well, because even at low levels they promote Fenton chemistry and other ROS-generating reactions. The traditional method for controlling metals is to add non-selective metal chelator, such as EDTA or EGTA, to isolation and recording solutions [21]. While convenient for most approaches, supplemental chelators confound the study of metal-mitochondria interactions in several ways: (i) to obtain appreciable free metal levels, excess metal must be added to overcome the chelator pool; (ii) calculation of free metal concentration relies on dissociation constants established in conditions different from commonly used mitochondrial buffers; (iii) adding one metal species can change the free levels of others by competitive binding, which requires time to equilibrate, so conditions will inevitably vary over course of the experiment in a manner that is difficult to predict. We avoided these problems by superfusing our mitochondria with a KCl-based recording solution that had been treated with Chelex 100, and then stored in Teflon-coated containers. The resulting metal-depleted solutions supported mitochondrial function as well as solutions

prepared traditionally with supplemental EDTA. The Chelex approach allowed us to add Ca^{2+} or Zn^{2+} in relative assurance that the amount added closely approximated the free concentration of metal. The stuck-down approach has the further advantage of washing away metals, EDTA, soluble proteins, organelle fragments, and non-specific metal binders that collect in the suspension. Therefore we circumvented problems stemming from contamination, competitive binding, and complex free metal calculations. Using mitochondria attached to glass cover-slips also affords the convenience of stimulus exchange and washout, which is not possible with mitochondrial suspension. Another advantage is the luxury of monitoring the dynamics of depolarization in individual mitochondria, which is impossible in cuvette-based experiments.

The Ca^{2+} effects observed here are generally consistent with past studies, and it is noteworthy that a number of these used stuck-down mitochondria to investigate Ca^{2+} -induced depolarization and mPT [16, 22–27]. This suggests that isolated mitochondria adhered to glass and studied by microscopy are not fundamentally different from, nor are they more prone to artifact than, mitochondria studied by more traditional methods. It has been hypothesized that once imported into the matrix, Ca^{2+} complexes with phosphate, and when the phosphate buffer capacity is exceeded, mitochondria are more likely to undergo mPT [28]. Also in agreement with previous reports is the Ca^{2+} data we obtained with mPT inhibitors. Indeed, cyclosporine A, Mg^{2+} and ADP are all recognized inhibitors of Ca^{2+} -induced mPT in mitochondria from various tissue sources [as reviewed by 12]. Ruthenium red is not a direct inhibitor of mPT, but protects against it by inhibiting Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter.

The data presented here are consistent with previous work from our group. Using the stuck-down approach, we observed that relatively high concentrations of zinc were necessary to cause depolarization that was slow and partial, and therefore inconsistent with mPT [19]. In our work with cultured neurons, we found that relatively small zinc loads sufficient to either stop mitochondrial trafficking [29], or eventually result in cell death [30] did not cause acute mitochondrial depolarization. However a number of groups have reported evidence for an mPT or mPT-like consequence of Zn^{2+} [10, 11, 31, 32]. Perhaps these different outcomes might be explained by an indirect or delayed effect of Zn^{2+} . For example, mPT might eventually occur if depolarization persists, or if Zn^{2+} provokes accumulation of reactive oxygen species. In such cases, Zn^{2+} would act as an upstream stimulus or perhaps a predisposing factor to mPT, rather than an immediate cause. More simply, Zn^{2+} -induced mPT might occur slowly over a time frame that exceeds our examination of the events during and immediately after a brief exposure to Zn^{2+} . Whatever the case, our data do not support a direct and acute role for Zn^{2+} in initiating mPT.

A related issue concerns the potency of Zn^{2+} as a mitochondrial inhibitor. Here we needed Zn^{2+} in the tens of micromolar to achieve marked depolarization, and we estimated a Zn^{2+} IC_{50} of 18.69 μM . Previous reports, including one of ours, suggested that nanomolar Zn^{2+} could depolarize mitochondria [11, 33]. Those studies used the traditional approach of mitochondria in bulk suspension in cuvette-based assays. In light of the present data and the issues discussed above, it seems in retrospect that those mitochondria may have been impacted by metals liberated from the chelator or soluble proteins upon addition of Zn^{2+} , i.e. the comparatively heightened vulnerability was due to a metal cocktail that included Zn^{2+} and probably Fe, Cu, Ca^{2+} , etc. In contemplating mitochondrial vulnerability to Zn^{2+} , it is useful to consider studies using isolated complexes and enzymes. A recent report found that Zn^{2+} inhibits NADH: Ubiquinone reductase (complex 1) with an IC_{50} of 10–50 μM by an unprecedented mechanism [34]. Other work on isolated enzyme complexes reports Zn^{2+} affinity for cytochrome *bcl* as high as 3 μM [35]. Still other evidence suggests that Zn^{2+} inhibits Cytochrome *c* oxidase (complex IV) with a K_i of 1–10 μM [36–38]. The mechanism

at complex IV is complicated, but appears to involve inhibition of proton pumping. Brown and colleagues [8] calculated a submicromolar K_i for Zn^{2+} inhibition of the α -ketoglutarate dehydrogenase enzyme of the Krebs cycle. Viewed together, these numbers basically agree in the 10^{-6} M range. Thus, it is perhaps unsurprising that the intact organelle can tolerate 10^{-5} M Zn^{2+} without catastrophic consequences.

Zinc's relative lack of potency raises a global question regarding the degree to which Zn^{2+} inhibition of mitochondria contributes to cell death in models of zinc toxicity. In resting cells free Zn^{2+} is kept exceedingly low, perhaps pico- or low nanomolar [39–41]. In conditions idealized for Zn^{2+} influx, neurons may accumulate Zn^{2+} at hundreds of nanomolar [42]. Accepting these numbers at face value, and leaving aside difficulties inherent in their calculation [43], it is unlikely that living cells experience prolonged cytosolic Zn^{2+} higher than 1 μ M, let alone the tens of micromolar necessary here to elicit frank depolarization. Certainly, extracellular $[Zn^{2+}]$ far lower than what we used here can be lethal to cells when the plasma membrane is circumvented with an ionophore [30]. Thus it is difficult to attribute Zn^{2+} cytotoxicity solely to its effects on m . Of course, most scenarios that raise cytosolic Zn^{2+} to toxic levels also involve generalized disruption of ion homeostasis and accumulation of reactive oxygen species [44–47]. An important and possibly synergistic contribution by Zn^{2+} , amidst a cluster of insults, seems a more tenable hypothesis.

There is evidence that induction of mPT occurs differently in mitochondria from different tissues. We chose liver mitochondria for this study, because their mPTP is well-documented and readily observed. In contrast, mitochondria isolated from brain do not readily manifest all aspects of mPTP, and in particular are not consistently modulated by CsA [48–50]. The main point is that liver mitochondria are regarded as the easiest model for showing classic, overt mPTP. If liver mitochondria do not recapitulate Zn^{2+} -induced mPTP and Zn^{2+} inhibition in general, then the assumption that other tissues are more sensitive is questionable.

The mechanism behind Zn^{2+} -induced depolarization appears to be inhibition of the electron transport chain, probably from an externally accessible site. This would account for the ease of reversibility, and might explain the slow progression given that mPTP would be expected to result in fast collapse of m . Our previous studies in brain mitochondria suspension showed that Zn^{2+} depolarization was readily reversed with EDTA, which is membrane impermeant and presumably does not enter the matrix. Others suggested that Zn^{2+} inhibits electron transport at the Cytochrome *bcl* complex [9], and were corroborated by crystallographic identification of Zn^{2+} binding sites in that complex [51]. Because block of the uniporter with ruthenium red gives robust protection against Ca^{2+} -induced depolarization, we conclude that the uniporter-mediated Zn^{2+} uptake (to the extent that it occurs) is not an important factor in Zn^{2+} -induced depolarization. Our previous work suggested that Zn^{2+} uptake in brain mitochondria can occur via the uniporter, as well as by other unidentified mechanisms [19]. The metallothioneins, a family of small Zn^{2+} binding proteins, may serve as chaperones that mediate mitochondrial Zn^{2+} import [52, 53]. However, this is improbable in our model, because extramitochondrial proteins are not expected to remain when mitochondria, adhered coverslip glass, are constantly rinsed with KCl-HEPES buffer. Other import pathways might include Zn^{2+} transporters, several which are known to localize to, and transport Zn^{2+} into, organelles. However, no mitochondrial Zn^{2+} transporter has been identified yet, and a study of the heart mitochondria proteome revealed no obvious candidates [54].

In closing, a large body of literature suggests that Zn^{2+} -toxicity involves a cluster of abnormalities affecting the bioenergetic state of the cell, including ROS accumulation,

inhibition of enzymes in glycolysis and the Krebs cycles, activation of oxidase enzymes, and inhibition of electron transport. However further experiments are necessary to determine which of these is critical and relevant to Zn^{2+} -induced toxicity *in vivo*.

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Abbreviations used

CsA	cyclosporine A
m	mitochondrial membrane potential
mPTP	mitochondrial permeability transition pore
TMRM	tetramethylrhodamine methyl ester perchlorate
Rh123	rhodamine 123
ROS	reactive oxygen species

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Figure 1. Different dynamics of Ca^{2+} - and Zn^{2+} -induced depolarization in isolated liver mitochondria. Representative traces depict changes in Rh123 fluorescence (F_{Rh123}) during 6-minute exposures to $1\mu\text{M}$ (A, E), $10\mu\text{M}$ (B, F), $30\mu\text{M}$ (C, G) and $100\mu\text{M}$ (D, H) Ca^{2+} or Zn^{2+} , respectively. Each trace represents a fluorescence signal from a single mitochondrion. A decrease in Rh123 signal reflects mitochondrial depolarization. Each panel shows the responses of about 20 mitochondria taken from an experiment where 40–80 mitochondria were sampled.

Figure 2.

Zn^{2+} -induced mitochondrial depolarization occurs in a concentration-dependent manner. Summary data of Ca^{2+} - and Zn^{2+} -induced Rh123 changes represented as the number of mitochondria that fully depolarized at the end of 6-min divalent cation (Me^{2+}) exposure (A). Percent decrease of Rh123 fluorescence at the end of 6-min treatments with different Zn^{2+} concentrations (B). The difference between maximum signal in each mitochondrion and background fluorescence was established as 100%. For panel (A) Data are the mean $\pm$ SE from at least 3 separate coverslips, where N is the total number of coverslips using mitochondria isolated from at least three different animals. 40–80 individual mitochondria were analyzed in each experiment. Statistical comparison of control conditions to Ca^{2+} or Zn^{2+} treatments using two-tailed t-test indicates that only 10 and 30 μM Ca^{2+} are different from controls, * $p < 0.001$. For the concentration response data in panel (B), individual mitochondria sampled from at least five coverslips were considered together for an N of 400 to 700 mitochondria for each condition.

Figure 3.

Ca^{2+} -induced depolarization is delayed by treatment with mPT inhibitors and uniporter blockade. Representative traces of mitochondria exposed for 6 minutes to $10 \mu M Ca^{2+}$ alone (A), $10 \mu M Ca^{2+}$ with CsA ($1 \mu M$) (B), and $30 \mu M Ca^{2+}$ with RR ($2 \mu M$) (C). In panels D and E, the percentage of mitochondria fully depolarized in each condition is plotted as a function of the duration of stimulus: $10 \mu M Ca^{2+}$ (D) or $30 \mu M Ca^{2+}$ (E), in the presence or absence of CsA, ADP with oligomycin (100 and $2 \mu M$, respectively) or RR. CsA, RR, and ADP + oligomycin were applied 2 minutes prior to, and during the 6-minute Ca^{2+} stimulus.

Figure 4.

Neither mPTP inhibitors, nor Ca²⁺-uniporter blockade prevents Zn²⁺-induced depolarization. Mean traces from a single coverslip of Rh123 changes in mitochondria with 6-minute exposure to either 10 μ M (A) or 30 μ M (B) Zn²⁺ in the presence or absence of cyclosporin A (CsA, 2 μ M). In panel (C), a summary graph of percent decrease in Rh123 fluorescence produced by 10 μ M (open bars) or 30 μ M (solid bars) Zn²⁺. Control (Zn²⁺ alone) values were compared to Zn²⁺-induced depolarization with ruthenium red (RR, 2 μ M), cyclosporin A (CsA, 1 μ M), in high Mg²⁺ buffer (5 mM) or ADP (100 μ M) in the presence of oligomycin (2 μ M). In each condition, drugs were applied 2 minutes prior to and during the Zn²⁺ stimulus. Data in (C) are the mean $\pm$ SE from at least 3 separate coverslips, where N is the total number of coverslips using mitochondria isolated from at least three different animals. We found no significant differences ($P > 0.05$) between Zn²⁺-treated (control) and Zn²⁺+ drug-treated mitochondria, as determined by unpaired two-tailed t-tests.

Figure 5.

Ca^{2+} , but not Zn^{2+} , causes rapid loss of mitochondrial calcein fluorescence. (A) Representative calcein micrographs were captured before and after a 6-minute exposure to either 30 μ M Ca^{2+} or Zn^{2+} . Arrows refer to individual mitochondria in different images. Individual traces of calcein fluorescence from mitochondria exposed to buffer alone (B), 30 μ M Ca^{2+} (C) or 30 μ M Zn^{2+} (D). Only mitochondria with robust TMRM fluorescence before metal exposure were selected for analysis. (E) Summary graph of percent mitochondria that completely lost calcein (defined as the number that lost 85% of starting fluorescence levels). Mitochondria exposed to Ca^{2+} exhibited greater fluorescence loss compared to control or Zn^{2+} -treated conditions. Representative traces in (B–D) represent calcein fluorescence of individual mitochondria. Data in (E) are the mean $\pm$ SE from at least 3 separate coverslips, where N is the total number of coverslips using mitochondria isolated from at least three different animals. Statistical comparison using two-tailed t-test of each condition to control indicates a significant difference only in the presence of Ca^{2+} , * $p < 0.001$.