ACTA ABP BIOCHIMICA POLONICA

Vol. 57, No 4/2010
435–441
on-line at: www.actabp.pl

Regular paper

Mitochondrial respiratory chain inhibitors modulate the metal-induced inner mitochondrial membrane permeabilization★

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To elucidate the molecular mechanisms of the protective action of stigmatellin (an inhibitor of complex III of mitochondrial electron transport chain, mtETC) against the heavy metal-induced cytotoxicity, we tested its effectiveness against mitochondrial membrane permeabilization produced by heavy metal ions Cd^{2+}, Hg^{2+}, Cu^{2+} and Zn^{2+}, as well as by Ca^{2+} (in the presence of P_i) or Se (in form of Na_2SeO_3) using isolated rat liver mitochondria. It was shown that stigmatellin modulated mitochondrial swelling produced by these metals/metalloids in the isotonic sucrose medium in the presence of ascorbate *plus* tetramethyl-*p*-phenylenediamine (complex IV substrates added for energization of the mitochondria). It was found that stigmatellin and other mtETC inhibitors enhanced the mitochondrial swelling induced by selenite. However, in the same medium, all the mtETC inhibitors tested as well as cyclosporin A and bongkrekic acid did not significantly affect Cu^{2+}-induced swelling. In contrast, the high-amplitude swelling produced by Cd^{2+}, Hg^{2+}, Zn^{2+}, or Ca^{2+} *plus* P_i was significantly depressed by these inhibitors. Significant differences in the action of these metals/metalloids on the redox status of pyridine nucleotides, transmembrane potential and mitochondrial respiration were also observed. In the light of these results as well as the data from the recent literature, our hypothesis on a possible involvement of the respiratory supercomplex, formed by complex I (P-site) and complex III (S-site) in the mitochondrial permeabilization mediated by the mitochondrial transition pore, is updated.

Keywords: zinc, cadmium, mercury, copper, selenite, stigmatellin, mitochondria, permeability transition pore

Received: 01 September, 2010; revised: 16 October, 2010; accepted: 12 November, 2010; available on-line: 19 November, 2010

INTRODUCTION

As it is well-known, mitochondria are main targets for such important environmental pollutants as heavy metals, which are found to be strong carcinogenic, genotoxic and cytotoxic (Stohs & Bagchi, 1995; Waalkes, 2003; Waisberg *et al*., 2003; Valko *et al*., 2005; Nordberg, 2009). However, mechanism(s) underlying heavy metal-induced mitochondrial dysfunction and cytotoxicity are not fully understood. As proposed, disturbance of mitochondrial electron transport or respiratory chain (mtETC) function and activation of various mitochondrial ion channels, in particular the mitochondrial permeability transition (MPT) pore, are the critical events in many disorders and pathological conditions. They are also involved in different types of cell death, including that produced by heavy metals (Zoratti & Szabo, 1995; Fontaine & Bernardi, 1999; Lemasters *et al*., 2009; Halestrap, 2009). The MPT pore can be defined as a voltage-dependent, non-selective high-conductance inner mitochondrial membrane channel of unknown molecular structure, which allows solutes of up to 1500 Da to pass freely in and out of mitochondria. The MPT pore opens under conditions of calcium overload and oxidative stress, and its opening is greatly enhanced by adenine nucleotide depletion, elevated phosphate, as well as the oxidized state of pyridine nucleotides (PN) and of critical dithiols in at least two discrete redox-sensitive sites, P- and S-site, the localization of which is still unknown (Fontaine & Bernardi, 1999; Petronilli *et al*., 2009).

Previously, we have studied the modulating action of various antioxidants, mtETC and MPT pore inhibitors on cytotoxic effects of heavy metal ions Cd^{2+}, Hg^{2+}, and Cu^{2+} on rat hepatoma AS-30D cells cultivated *in vitro*. We found that all these metal ions produced significant changes in mitochondrial functions and in the intracellular generation of reactive oxygen species (ROS). It was also shown that the increased ROS level alone was not sufficient to induce apoptotic and/or necrotic decay of these cells. In the case of Cd^{2+} and Hg^{2+}, additional factor(s) must have been present that was/were responsible for their cytotoxic action; most likely, it was the blockage of the mtETC (Belyaeva *et al*., 2008). Notably, the Cd^{2+}-induced death of AS-30D cells was accompanied by an increased ROS formation at the level of mtETC complex III and opening of the MPT pore (Belyaeva *et al*, 2006). Moreover, stigmatellin, a potent. mtETC complex III inhibitor, was found to be one of the strongest protectors against the Cd^{2+}-induced cytotoxicity, in addition to *N*-acetylcysteine and two MPT pore inhibitors, cyclosporin A (CsA) and bongkrekic acid (BKA).

To elucidate molecular mechanisms of the protective action of stigmatellin against the heavy metal-induced

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★Part of this work was presented in the poster form at the 16th European Bioenergetics Conference (Warsaw, 2010); abstract in *Biochim Biophys Acta*, **1797** (Suppl): 79 (2010).

Abbreviations: ANT, adenine nucleotide translocase Asc, ascorbate; BKA, bongkrekic acid; BSA, bovine serum albumin; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CsA, cyclosporin A; DNP, 2,4-dinitrophenol; DTT, dithiothreitol; Glu, glutamate; Mal, malate; MCU, mitochondrial Ca^{2+} uniporter; mtETC, mitochondrial electron transport chain; MPT, mitochondrial permeability transition; P_i, inorganic phosphate; PN, pyridine nucleotides; Rh123, rhodamine 123; RLM, rat liver mitochondria; ROS, reactive oxygen species; RR, Ruthenium Red; Ru-360, Ruthenium-360; $\Delta\Psi_{mito}$, mitochondrial transmembrane potential; Succ, succinate; TMPD, tetramethyl-*p*-phenylenediamine

cytotoxicity, we tested effectiveness of different mtETC and MPT pore inhibitors against mitochondrial membrane permeabilization produced by heavy metal ions Cd^{2+}, Hg^{2+}, Cu^{2+}, and Zn^{2+}, as well as by Ca^{2+} (in the presence of P_i) or Se (added as Na_2SeO_3), using isolated rat liver mitochondria (RLM) as a model system. As result, significant distinctions in the action of these metals/metalloids on mitochondrial functions were revealed.

MATERIALS AND METHODS

Chemicals. Most reagents, including stigmatellin, Na_2SeO_3, and chloride salts of the metals tested, were obtained from Sigma Aldrich Co. (St. Luis, MO, USA). CsA was from Novartis (Basel, Switzerland). Other chemicals were of the highest purity commercially available.

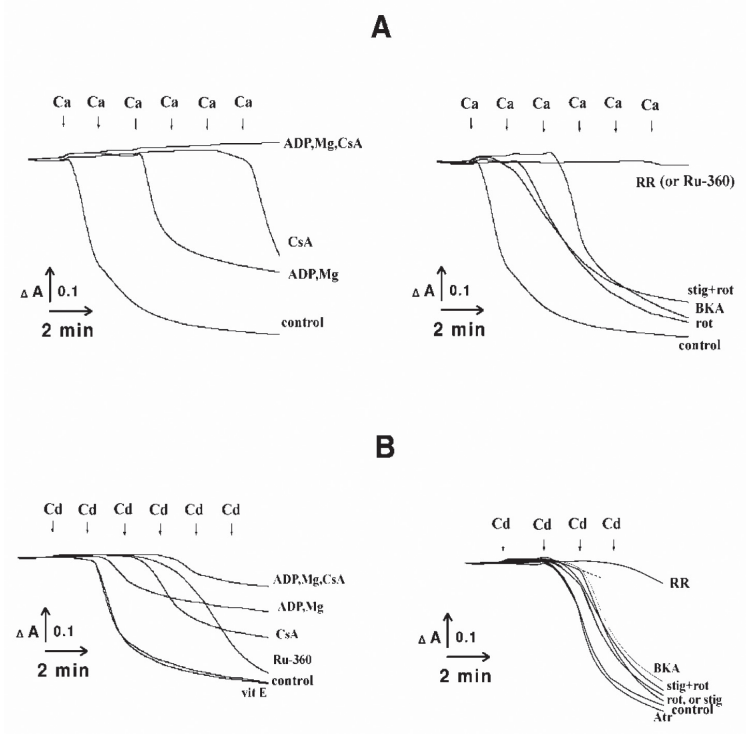
Preparation of rat liver mitochondria. Rat liver mitochondria were prepared by differential centrifugation after homogenization in a mannitol-sucrose medium containing 1 mM EGTA and 0.5% BSA (bovine serum albumin) according to Allhire *et al.* (1985). Mitochondria were washed two times using a medium without EGTA and BSA. Finally they were suspended in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM Hepes/Tris, pH 7.4. Most experiments were also repeated on mitochondria isolated using a homogenization medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4 and 0.5 mM EGTA/Tris, while EGTA was omitted in the washing medium, as described previously (Belyaeva *et al.*, 2002). The results obtained using both isolation media were practically the same. Protein content was measured by the biuret method with BSA as standard.

Mitochondrial function assays. Routine assays of mitochondrial functions such as determination of mitochondrial volume by measuring absorbance changes at 540 nm and monitoring oxygen consumption polarographically using a Clark-type electrode and/or the OROBOROS technique, have been described before (Belyaeva *et al.*, 2001; 2002; Belyaeva & Saris, 2008). Changes in the redox state of PN were estimated using NAD(P)H fluorescence (excitation, 340 nm; emission, 460 nm). The mitochondrial transmembrane potential ($\Delta\Psi_{mito}$) was estimated using fluorescence of Rhodamine 123 (0.25 μM; excitation, 503 nm; emission, 527 nm). The increase in Rh123 fluorescence shows the decrease in $\Delta\Psi_{mito}$. In some cases, the fluorescent probe safranine O was used to monitor $\Delta\Psi_{mito}$ changes. For composition of the media and experimental details, see figure legends. The results shown are representative or average of a series of at least three independent experiments. Statistical analysis was performed using two-tailed Student's *t*-test. A P value < 0.05 was considered statistically significant.

RESULTS

It is considered that the mitochondrial swelling in isotonic sucrose medium is a marker of the MPT pore involvement in the mitochondrial membrane permeabilization (Zoratti & Szabo, 1995). It is also known that Ca^{2+} *plus* P_i are strong inducers of the classical or regulated MPT pore, i.e., activated by Ca^{2+} and inhibited by CsA and Mg^{2+} (Zoratti & Szabo, 1995; He & Lemasters, 2002). Previously, using isolated RLM, we (Belyaeva *et al.*, 2001; 2002; 2004a) and other authors (Dorta *et al.*, 2003) revealed that Cd^{2+} was a potent inducer of the nonselective MPT pore operated not only in its regulated mode (see above) but also in its unregulated mode that was Ca^{2+}-independent and CsA-insensitive, like in the case of high Hg^{2+} (He & Lemasters, 2002). Besides, we found that mtETC inhibitors such as rotenone and stigmatellin affected the Cd^{2+}-produced mitochondrial dysfunction in a way indicating involvement of mtETC complexes in the MPT pore modulation and in the Cd^{2+}-induced mitochondrial membrane permeabilization (Belyaeva & Korotkov, 2003; Belyaeva *et al.*, 2004b; Belyaeva & Saris, 2008).

In this work we studied the action of different MPT pore and mtETC inhibitors on mitochondrial swelling induced by the metals/metalloids under test in isotonic sucrose medium in the presence of Asc *plus* TMPD, i.e., mtETC complex IV substrates. These respiratory substrates were added to energize mitochondria under conditions when respiratory complexes I, II, and III might have been inhibited by added heavy metal ions, e.g., Cd^{2+}. We showed that under these conditions rotenone and/or stigmatellin exhibited modulating effects on the swelling induced by the metals/metalloids tested. In particular, rotenone and/or stigmatellin significantly, though to a different extent, depressed the high-amplitude swelling produced by Ca^{2+} (*plus* P_i), Cd^{2+}, Zn^{2+} or Hg^{2+} (Fig. 1). As expected, ruthenium red (RR), ruthenium-360 (Ru-360), BKA, CsA and ADP *plus* Mg^{2+} were also strongly inhibitory (Fig. 1). It should be noted that stigmatellin produced a significant effect also in the absence of rotenone. As evident, mitochondrial membrane permeabilization produced by Zn^{2+}, Cd^{2+} or Hg^{2+} was more strongly depressed by ADP *plus* Mg^{2+} than by CsA, whereas CsA was a stronger inhibitor than ADP *plus* Mg^{2+}



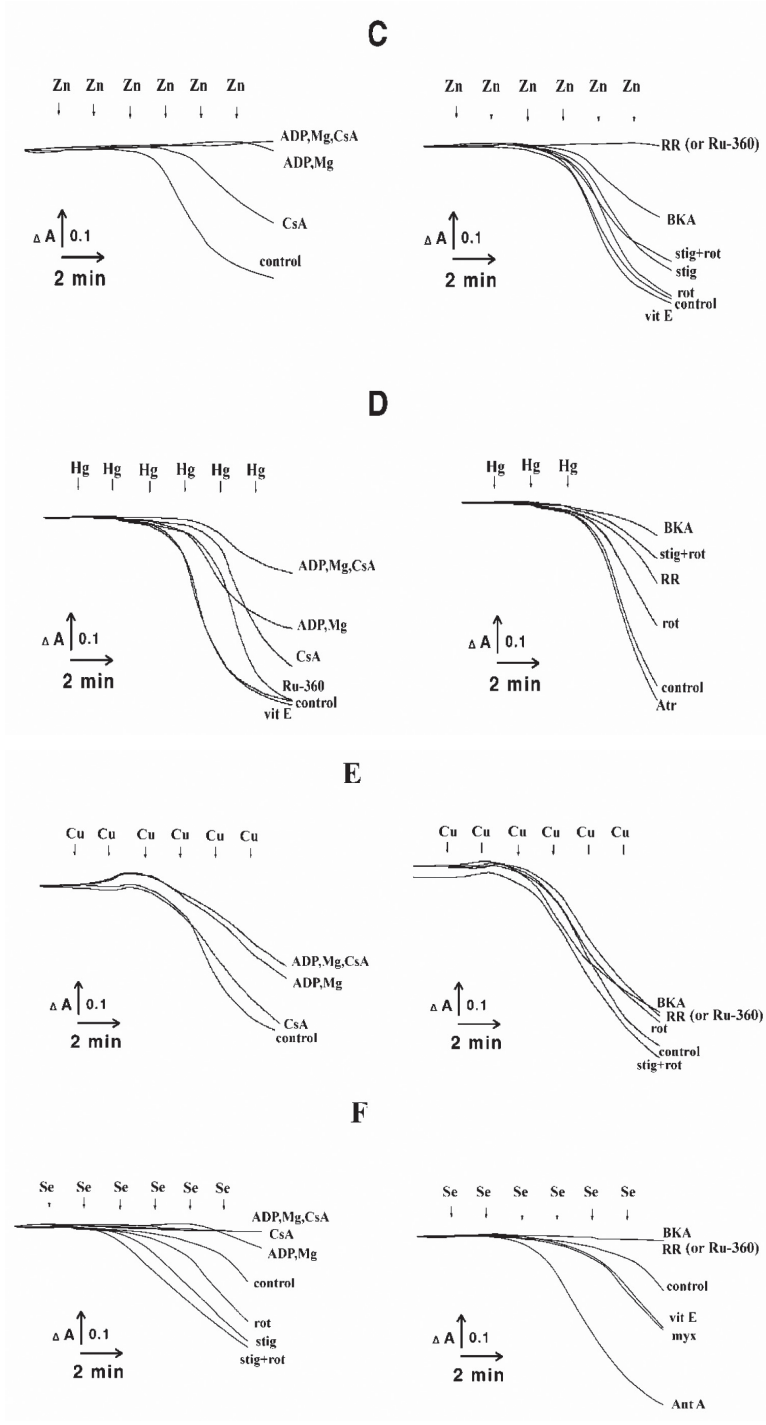


Figure 1. Effects of Ca^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and sodium selenite on the mitochondrial swelling
Mitochondria (1 mg protein/ml) were incubated in a medium containing 250 mM sucrose, 5 mM Tris/HCl (pH 7.4), 1 mM Asc, 20 μM TMPD and oligomycin — 1 μg/ml. Additions of Ca^{2+} (50 μM), Cd^{2+} (2.5 μM), Zn^{2+} (5 μM), Hg^{2+} (10 μM), Cu^{2+} (10 μM), or Na_2SeO_3 (15 μM) are indicated by arrows. The concentrations of other additions were: CsA, 1 μM; BKA, 7 μM; ADP, 0.5 mM; Mg^{2+}, 0.5 mM; RR, 7 μM; Ru-360, 10 μM; rotenone, 1 μM; stigmatellin, 1 μM; myxothiazol, 1 μM; antimycin A, 1 μM; atractyloside, 20 μM; vitamin E, 0.5 mM. The results are representative for a series of four independent experiments.

in the case of swelling induced by Ca^{2+} *plus* P_i or selenite. Notably, in all cases the combination of CsA and ADP *plus* Mg^{2+} exhibited the maximal protective effect (Fig. 1). In addition, it was found that the protective action of the MPT and mtETC effectors against mitochondrial swelling was overcome by increase of the metal load. It can also be noted that stigmatellin and other mtETC inhibitors strongly enhanced mitochondrial swelling produced by selenite (Fig. 1F). It is noteworthy that, in the same medium and under the same conditions, the mtETC inhibitors used as well as RR (or Ru-360), BKA and CsA affected the Cu^{2+}-induced swelling only weakly, whereas ADP *plus* Mg^{2+} exerted a stronger inhibitory effect (Fig. 1E).

As it is well known, changes in $\Delta\Psi_{mito}$, the redox status of mitochondrial PN and disturbance of the respiratory function could be both the cause and the consequence of the mitochondrial membrane permeabilization (Zoratti & Szabo, 1995). With the aim to understand the reason of the differences in the action of MPT and mtETC inhibitors on membrane permeabilization as well as to underscore the cause/consequence relationships underlying the mitochondrial dysfunction produced by the metal/metalloids under test, we compared the effects of Ca^{2+}, Cd^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and selenite on the PN redox status (Fig. 2), $\Delta\Psi_{mito}$ (Fig. 3) and respiration of isolated RLM (Fig. 4) and their sensitivity (if so) to CsA. As result, we found significant distinctions in the action of these metals/metalloids on all these mitochondrial bioenergetics parameters. In particular, using the KCl respiratory assay medium (for the content of which see legend to Fig. 2), we observed that the action of Zn^{2+} on the mitochondrial function resembled that of Cd^{2+} as described previously (Belyaeva *et al.*, 2002; Belyaeva & Korotkov, 2003; Belyaeva *et al.*, 2004a; 2004b; Belyaeva & Saris, 2008) and in the present study. We found that a sharp oxidation of PN (Fig. 2B and C), $\Delta\Psi_{mito}$ dissipation (Figs. 3B and C), the respiratory dysfunction (Fig. 4) and matrix swelling produced by both these metals were strongly inhibited by CsA. Nevertheless, a significant decrease of $\Delta\Psi_{mito}$ occurred even in the presence of CsA in case of both Zn^{2+} and Cd^{2+}. Importantly, the protective action of CsA against the harmful effects of Cd^{2+} or Zn^{2+} was eliminated by increase of the heavy metal load. In turn, we found that $\Delta\Psi_{mito}$ dissipation induced by pulses of selenite in the KCl medium was completely depressed by CsA (Fig. 3D), the same being true for Ca^{2+} (Fig. 3A). In addition, CsA restored Ca^{2+} uptake capacity that had been disturbed by selenite treatment (not shown). However, a strong sustained activation of the basal respiration rate of the mitochondria found in the presence of selenite (see Fig. 4A, trace 3) was only in part decreased by CsA (not shown). Besides, mitochondrial swelling induced by selenite in the KCl medium was also only partially susceptible to CsA, in opposite

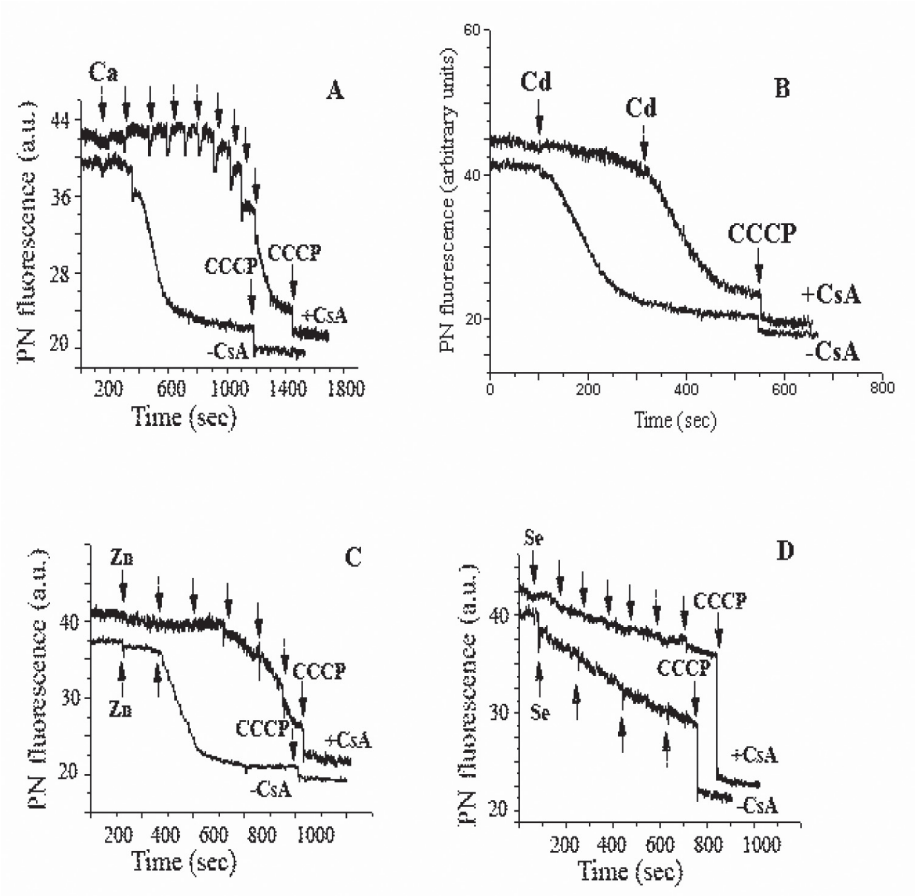


Figure 2. Effects of Ca^{2+}, Cd^{2+}, Zn^{2+} and sodium selenite on the redox state of mitochondrial pyridine nucleotides

Mitochondria (0.5 mg protein/ml) were incubated in a medium containing 100 mM KCl, 3 mM KH_2PO_4, 3 mM $MgCl_2$, 20 mM Hepes (pH 7.3), 5 mM Glu, 5 mM Mal and oligomycin — 1 μg/ml. The additions of Ca^{2+} (50 μM), Cd^{2+} (5 μM), Zn^{2+} (5 μM), or Na_2SeO_3 (5 μM) and CCCP (1 μM) are indicated by arrows. CsA was 1 μM. The results are representative for a series of three independent experiments.

to the Ca^{2+}-induced swelling or the respiratory dysfunction, which were completely eliminated by CsA. Finally, we observed that the effect of selenite on PN redox status in isolated RLM (Fig. 2D) differed strongly not only from that of Cd^{2+} (Fig. 2B) or Zn^{2+} (Fig. 2C) but also from that of Ca^{2+} (Fig. 2A). As seen, after selenite pulses there was no strong and rapid decrease in the PN autofluorescence (eliminated by addition of CsA) as it was observed in the case of the metal ions mentioned above. Instead, a moderate and continuous PN oxidation occurred in the case of selenite that were significantly retarded in the presence of CsA (Fig. 2). We also found that under our conditions $\Delta\Psi_{mito}$ decrease, PN oxidation and respiratory dysfunction produced by Hg^{2+} or Cu^{2+} in isolated RLM were insensitive to CsA (not shown).

DISCUSSION

The present study was aimed to a further elucidation of molecular mechanisms of the metal-induced mitochondrial inner membrane permeabilization as well as the protective action of stigmatellin against the heavy metal-induced cytotoxicity as shown previously in two rat cell lines, hepatoma AS-30D (Belyaeva *et al.*, 2006) and PC12 cells (unpublished). The evidence obtained agrees well with data found before by us and others and adds new important information about mechanism(s) of the metals/metalloids toxicity. Although involvement of MPT pores in the mechanism(s) of mitochondrial dysfunction induced by both Zn^{2+} (Wudarczyk *et al.*, 1999; Jiang *et al.*, 2001; Dineley *et al.*, 2003; Bossy-Wetzel *et al.*, 2004; Gazaryan *et al.*, 2007; Devinney *et al.*, 2009) and selenite (Kim *et al.*, 2002; 2003; Shilo *et al.*, 2003; Shilo & Tirosh, 2003) was suggested a long time ago, it was still under debate in the former case (Dineley *et al.*, 2003; Devinney *et al.*, 2009). In particular, Dineley and co-authors argued against the participation of the MPT pore in Zn^{2+}-produced primary depolarization of brain (Dineley *et al.*, 2003) and liver mitochondria (Devinney *et al.*, 2009). Our results also point to the existence of both the CsA-sensitive and CsA-insensitive components in Zn^{2+}(Cd^{2+})-produced depolarization and swelling of isolated RLM as well as to a marked similarity of the Zn^{2+} and Cd^{2+} action on mitochondrial functions under our conditions (Figs. 1–4). In addition, some authors argued against the MPT pore participation in mechanisms of Cd^{2+} toxicity in rat liver (Al-Nasser, 2000) or kidney mitochondria (Lee *et al.*, 2005a; 2005b), mainly on the basis of the insensitivity of Cd^{2+} effects to CsA and/or BKA in their experimental models. However, data obtained here indicate that the protection by CsA, BKA and RR can be prevented by increase of the metal load both in the case of Cd^{2+} and other heavy metal ions used (Fig. 1). Besides, the Cd^{2+} effects depend on the substrate used for mitochondrial energization (Fig. 4B) as well as on medium composition. Moreover, they strongly depend not only on the concentration of the metal ion but also on the mode of its addition to the mitochondria. In particular, our data show that in the isotonic sucrose medium low Cd^{2+} pulses induced the high-amplitude RLM swelling (Fig. 1B), while the swelling was negligible if a single high Cd^{2+} (50–70 μM) pulse was added (unpublished), indicating different modes of action of this heavy metal ion.

Notably, the protective action of rotenone and stigmatellin against the mitochondrial swelling evoked by Ca^{2+}, Hg^{2+} or Zn^{2+} found in this study (in addition to the previous data with Cd^{2+}, see above) points to the involvement of the mtETC in the metal-induced mitochondrial membrane permeabilization (Fig. 1A–D). In turn, the promoting effect of stigmatellin (as well as

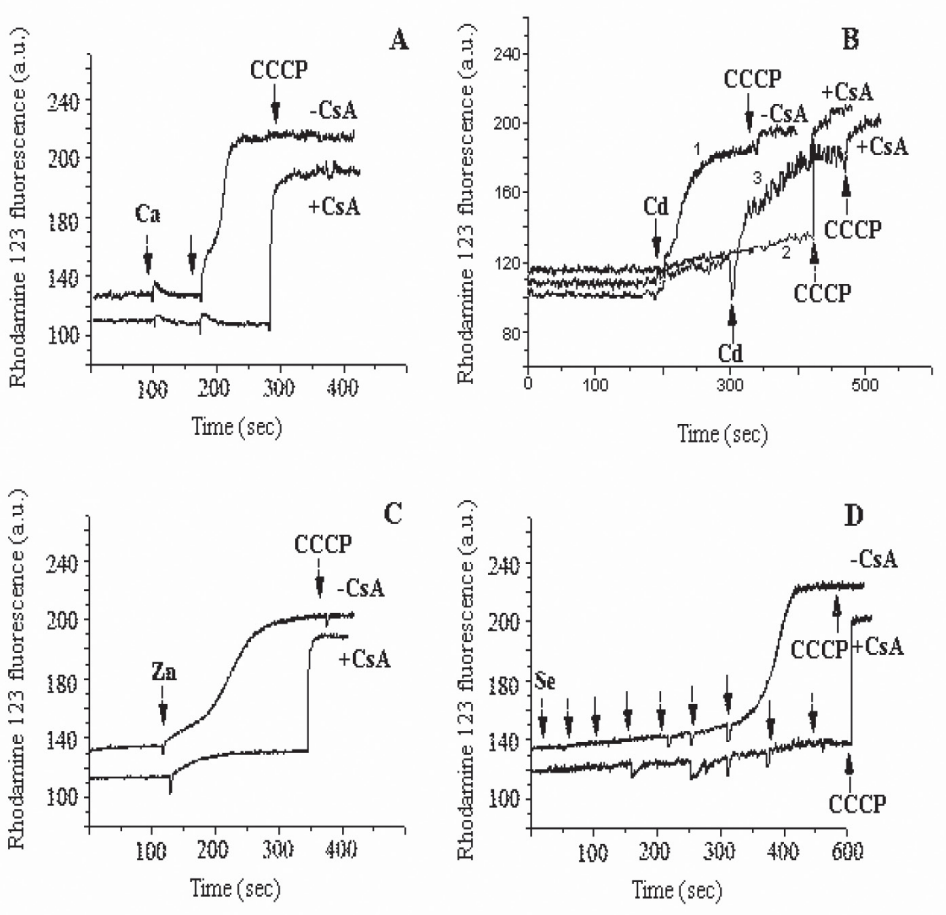


Figure 3. Effects of Ca^{2+}, Cd^{2+}, Zn^{2+} and sodium selenite on the mitochondrial transmembrane potential

The additions of Ca^{2+} (100 μM), Cd^{2+} (10 μM), Zn^{2+} (10 μM) or Na_2SeO_3 (10 μM) and CCCP (1 μM) are indicated by arrows. The increase in Rh123 fluorescence shows the decrease in $\Delta\Psi_{mito}$. Other conditions as in Fig. 2. In panel **(B)** the following additions were made: trace 1, one Cd^{2+} pulse; trace 2, one Cd^{2+} pulse in the presence of CsA; trace 3, two Cd^{2+} pulses in the presence of CsA.

rotenone, myxothiazol, and antimycin A) on the mitochondrial swelling in the presence of selenite (Fig. 1F) indicates a crucial difference in the mechanism(s) of the induction of the mitochondrial permeabilization by selenite and the metal ions. The data on selenite action on the PN redox status of isolated RLM in the absence and in the presence of CsA (Fig. 2D) also support this conclusion. Markedly, the absence of significant action of mtETC inhibitors on the Cu^{2+}-induced mitochondrial membrane permeabilization found in the present work (Fig. 1E) points to a different mechanism of this metal ion toxicity compared to that of other heavy metal ions tested. Besides, a complete insensitivity of Cu^{2+} effects to RR (or Ru-360) and CsA (in opposite to ADP *plus* Mg^{2+}) found here suggests that under our conditions Cu^{2+} acts predominately from the external site, affecting oxidative phosphorylation and the membrane permeability, in support to data obtained by Leblondel and Allain (1984). Thus, a likely target of this metal ion could be the adenine nucleotide translocase (ANT) (Zazueta *et al.*, 1998; Garcia *et al.*, 2000) and/or a critical external dithiol described recently (Petronilli *et al.*, 2009). Our results also show that Cd^{2+}, Hg^{2+} and may be also Zn^{2+} seemingly act *via* both the external and the internal set of sites, i.e., located at the cytosolic or the matrix sides of the inner membrane. These sites involve the critical pore-regulating dithiols and the Ca^{2+}(or Me^{2+})-binding sites, most likely His sites (Petronilli *et al.*, 2009). A direct participation of ANT and/or P_i carrier(s) and CyP-D (Halestrap, 2009) as well as the mtETC components, namely complex I (Fontaine & Bernardi, 1999) and/or complex III (Belyaeva, 2004; Belyaeva *et al.*, 2004b), is proposed in agreement with data found before by us and others (Chavez & Holguin, 1988; Chavez *et al.*, 1989; Dierks *et al.*, 1990a; 1990b; Koike *et al.*, 1991; Zoratti & Szabo, 1995; Schroers *et al.*, 1997; Zazueta *et al.*, 2000; Belyaeva *et al.*, 2001; 2004a; He & Lemasters, 2002; Belyaeva & Korotkov, 2003). In turn, it seems that Ca^{2+} induces the permeabilzation mainly *via* the internal set of sites (Fig. 1A). However, its action *via* the external set of sites under certain conditions is also proposed (see Petronilli *et al.*, 2009 and references therein). Unfortunately, up-to-date, the exact localization of these Me^{2+}-binding sites, critical for the MPT induction, as well as the P- and S-sites of the MPT pore (Fontaine & Bernardi, 1999) cannot be identified, despite several speculations (see Belyaeva *et al.*, 2004b; and references therein). In particular, we have speculated that both respiratory complexes I and III might be involved in the mitochondrial membrane permeabilization promoted by Cd^{2+} and/or Ca^{2+} *plus* P_i. We also hypothesized that complex I of the respiratory chain might constitute the P-site while complex III — the S-site of the MPT pore, and depending on conditions and cell type, either one or both complexes could be involved in triggering the MPT pore assembly. Moreover, complex I (P-site) and complex III (S-site) might constitute not only critical Me^{2+}-binding sites but also the main loci for ROS generation that were instrumental in oxidation of critical thiol groups and the MPT pore opening. We proposed that the aforementioned Me^{2+}-binding sites were most likely located on (i) the pathway of reverse electron transfer from succinate to NAD^+ (complex I) and (ii) cytochrome *b* somewhere in the vicinity of heme b_L and close to the stigmatellin binding site (complex III) (Belyaeva, 2004; Belyaeva *et al.*, 2004b). Later on we obtained further evidence confirming our speculations for the localization of P and S redox sites at complexes I and III, respectively (Belyaeva & Saris, 2008). In particular, we found that with Glu *plus* Mal (i.e., sub-

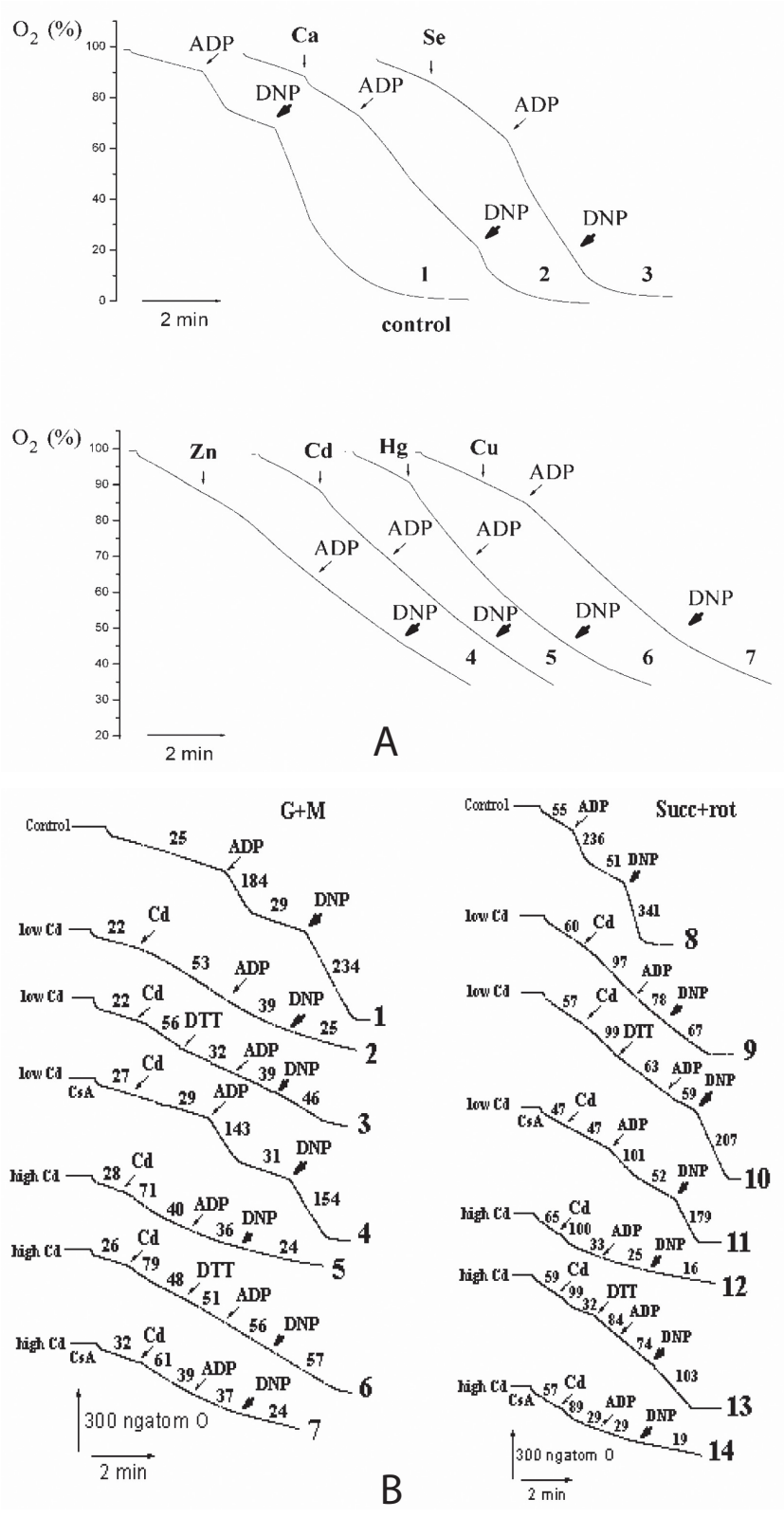


Figure 4. Effects of the metals/metalloids on mitochondrial respiration
Mitochondria (1 mg protein/ml) were incubated in the same medium as in Fig. 2 except that oligomycin was omitted. The substrates (present in the medium before addition of mitochondria) were: 5 mM Glu plus 5 mM Mal in **(A)** and in the left panel of **(B)**, and 5 mM succinate plus 1 μM rotenone in the right panel of **(B)**. The additions of Cd^{2+} (A: 10 μM; B: 10 or 70 μM, i.e. low and high Cd, respectively), Ca^{2+} (150 μM), Zn^{2+}, Hg^{2+}, Cu^{2+} or Na_2SeO_3 (10 μM each), ADP (100 μM), DNP (30 μM) and DTT (1 mM) are indicated by arrows. CsA was 1 μM. The results are representative for a series of four independent experiments.

strates of respiratory complex I) the respiration in the presence of DNP (which was strongly inhibited by Cd^{2+}) was weakly sensitive to dithiothreitol (DTT) in contrast to that obtained with Succ *plus* rotenone (Fig. 4B; see also Belyaeva & Korotkov, 2003; Belyaeva & Saris, 2008). Moreover, the results of the present study point to a critical involvement of respiratory complexes I and III in the metal ion-induced mitochondrial inner membrane permeabilization. Altogether, this supports our hypothesis that a mtETC supercomplex formed by complex I (P-site) and complex III (S-site), is likely to be involved in the mitochondrial membrane permeabilization mediated by the MPT pore and that complex III could contain the critical external dithiol (see above). It also seems that supercomplex I–III could be the key component of the regulated MPT pore, while complex III alone is likely involved in the unregulated MPT pore assembly. Some of these views agree well with data obtained by others during the latest time (He & Lemasters, 2005; Petronilli *et al.*, 2009; Lenaz *et al.*, 2010). A more detailed description of our model updated according to the present views in the field, including as a thorough review of the supporting data in the literature, is in preparation.

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