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Bivalent Metal Ions Modulate Cd^{2+} Effects on Isolated Rat Liver Mitochondria[1]

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We have studied Cd^{2+}-induced effects on mitochondrial respiration and swelling in various media as a function of the [Cd^{2+}] in the presence or absence of different bivalent metal ions or ruthenium red (RR). It was confirmed by monitoring oxygen consumption by isolated rat liver mitochondria that, beginning from 5 μM, Cd^{2+} decreased both ADP and uncoupler-stimulated respiration and increased their basal respiration when succinate was used as respiratory substrate. At concentrations higher than 5 μM, Cd^{2+} stimulated ion permeability of the inner mitochondrial membrane, which was monitored in this study by swelling of both nonenergized mitochondria in 125 mM KNO_3 or NH_4NO_3 medium and succinate-energized mitochondria incubated in a medium containing 25 mM K-acetate and 100 mM sucrose. We have found substantial changes in the above-mentioned Cd^{2+} effects on mitochondria treated in sequence with 100 μM of Ca^{2+}, Sr^{2+}, Mn^{2+} or Ba^{2+}(Me^{2+}) and 7.5 μM RR, as well as the alterations in Cd^{2+} action on the uptake of $^{137}Cs^{+}$ by succinate-energized mitochondria in the presence or absence of valinomycin in acetate medium (50 mM Tris-acetate and 140 mM sucrose) with or without Ca^{2+} or RR. The evidence obtained indicate that Ca^{2+} exhibits a synergestic action on all Cd^{2+} effects examined, whereas Sr^{2+} and Mn^{2+}, conversely, are antagonistic. In the presence of RR, the Cd^{2+} effects on respiration [stimulation of State 4 respiration and inhibition of 2,4-dinitrophenol (DNP)-uncoupled respiration] still exist, but are observed at concentrations of cadmium more than one order higher; the inhibition of State 3 respiration by Cd^{2+}, conversely, takes place under even lower cadmium concentrations than those determined without RR in the medium. In addition, RR added simultaneously with cadmium in the incubation medium prevents any swelling in the nitrate media, but induces an increment both in Cd^{2+}-stimulated swelling and $^{137}Cs^{+}$ (analog of K^{+}) uptake in the acetate media. For the first time, we have shown that Cd^{2+}-induced swelling in all media under study is susceptible to cyclosporin A (CSA), a high-potency inhibitor of the mitochondrial permeability transition (PT) pore. The observations are interpreted in terms of a dual effect of cadmium on respiratory chain activity and permeability transition.

KEY WORDS: Cd^{2+}; bivalent metal ions; ruthenium red; mitochondrial respiration; mitochondrial swelling; uptake of ^{137}Cs; mitochondrial permeability transition; ion transport; inner mitochondrial membrane.

INTRODUCTION

It has been known for a long time that Cd^{2+} is an uncoupling agent in mammalian mitochondria (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1962; Brierley and Murer, 1964). As obtained previously, low concentrations of Cd^{2+} stimulate State 4 respiration of isolated mitochondria from liver, kidney, and some other tissues and organs (Diamond and Kench, 1974; Mustafa and Cross, 1974; Sato *et al.*, 1978; Jarvisalo *et al.*, 1980; Chavez *et al.*, 1985; Cameron *et al.*, 1986; Skul'skii *et al.*, 1988).

[1] Key to abbreviations: DNP, 2,4-dinitrophenol; P_i, inorganic phosphate; EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; RR, ruthenium red; CSA, cyclosporin A; PT, mitochondrial permeability transition; $\Delta\psi_{mito}$ mitochondrial membrane potential.

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It has also been established that cadmium increases ion permeability of the inner mitochondrial membrane (Brierley, 1967; Blondin *et al.*, 1969; Nogami, 1979a; Sanadi *et al.*, 1981; Rasheed *et al.*, 1984; Korotkov *et al.*, 1998). On the other hand, Cd^{2+} decreases uncoupler-stimulated mitochondrial respiration (Kisling *et al.*, 1987; Skul'skii *et al.*, 1988; Miccadei and Floridi, 1993) and inhibits particular respiration chain enzymes such as the cytochrome bc_1 complex (Miccadei and Floridi, 1993), succinate-dehydrogenase (Jay *et al.*, 1991), NADH-dependent enzymes (Toury *et al.*, 1985; Cameron *et al.*, 1986), as well as cytochrome *c* oxidase (Prasada Rao, 1983; Muller and Stacey, 1988), although the last one only *in vivo* (Toury *et al.*, 1985). A rise of Cd^{2+} concentration in the incubation medium results both in the further inhibition of mitochondrial respiration and in the increase of matrix volume caused by Cd^{2+}-induced swelling of mitochondria (Jacobs *et al.*, 1956; Nogami, 1979b; Rasheed *et al.*, 1984). State 3 respiration of mitochondria is also diminished by Cd^{2+} and is extremely sensitive to an increment of cadmium concentration in the medium (Ogata *et al.*, 1978; Sato *et al.*, 1978; Cameron *et al.*, 1986; Kisling *et al.*, 1987; Skulskii *et al.*, 1988). Similar results were found on isolated hepatocytes (Muller and Ohnesorge, 1984; Muller, 1986; Liu and Liun, 1990) and the investigations on isolated cells added further evidence of the role of mitochondria as primary targets in Cd^{2+}-induced cytotoxicity. Moreover, it has been shown that cadmium induces a drop in the mitochondrial membrane potential of isolated mitochondria (Chavez *et al.*, 1985) and mitochondria *in situ* (Martel *et al.*, 1990; Koizumi *et al.*, 1994). In addition, as demonstrated in many studies both *in vitro* and *in vivo*, cadmium produces oxidative stress and causes lipid peroxidation (for a comprehensive review, see Rikans and Yamano, 2000). At present, although the role of oxidative damage in cadmium-induced hepatotoxicity is still controversial (Casalino *et al.*, 1997), GSH depletion, as well as lipid peroxidation per se, are not considered to be the major mechanisms of the toxicity of cadmium (Rikans and Yamano, 2000).

As suggested for many years, the most significance in mitochondrial Cd^{2+} toxicity belongs to its uncoupling action on oxidative phosphorylation; moreover, it was presumed that the effects caused by cadmium may have resulted from changes in the fluidity and permeability of the mitochondrial membrane (Liu and Liun, 1990). Later it was proposed that Cd^{2+} exerts its toxic effects mainly because it blocks mitochondrial electron transport chain by impairment of electron flow through the cytochrome bc_1 complex (Miccadei and Floridi, 1993). In turn, the uncoupling effect of Cd^{2+} on oxidative phosphorylation, as hypothesized by others, is due to the acceleration of H^+ influx through the P_i/H^+ symporter activated by Cd^{2+} (Koike *et al.*, 1991). There is also a supposition that Cd^{2+}-induced cellular acidification plays an important role in the initiation of deteriorative processes in mitochondria *in situ* (Koizumi *et al.*, 1994). In addition, it is generally considered that most of Cd^{2+} effects, which provoke the mitochondrial dysfunction, are explained by interactions of cadmium with different types of protein thiol groups on both sides of the inner mitochondrial membrane (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1962, 1963; Yagi and Hatefi, 1984; Rasheed *et al.*, 1984; Chavez *et al.*, 1985; Skul'skii *et al.*, 1988). The evidence of the involvement of cadmium as an external effector in the regulation of energy metabolism in rat liver mitochondria are also described in the literature (Brand *et al.*, 1988; Brand, 1997). In spite of the abundance of information on the issue, the exact mechanism of Cd^{2+}-induced dysfunction in rat liver mitochondria is still uncertain.

As found earlier, different divalent cations (including Mn^{2+} and Sr^{2+}) decreased (Hunter and Ford, 1955; Jacobs *et al.*, 1956; Caplan and Carafoli, 1965) while RR eliminated (Saris and Jarvisalo, 1977; Rasheed *et al.*, 1984; Chavez *et al.*, 1985; Skul'skii *et al.*, 1988; Koike *et al.*, 1991) some Cd^{2+} effects on respiration and swelling of isolated mitochondria. However, Cd^{2+} action on mitochondrial respiration in the presence of bivalent metal ions or RR in an incubation medium were not tested previously over a wide range of Cd^{2+} concentrations. It is not also clear, why some bivalent metal ions counteract the deleterious effect of Cd^{2+} on oxidative phosphorylation. It is widely accepted now, that Cd^{2+} (Jarvisalo *et al.*, 1980; Rasheed *et al.*, 1984; Chavez *et al.*, 1985), like Ca^{2+}, is transported into mitochondria via a Ca^{2+}-uniporter (Nicholls and Akerman, 1982; Gunter *et al.*, 1994, 1998). Transport of Sr^{2+} (Carafoli, 1965), Mn^{2+} (Drahota *et al.*, 1965; Kohji *et al.*, 1985) and Ba^{2+} (Drahota *et al.*, 1965; Lucaks and Fonnyo, 1985) follow the selectivity series $Ca^{2+} > Sr^{2+} > Mn^{2+} > Ba^{2+}$ (Vainio *et al.*, 1970). There is also evidence that these divalent metal ions are involved in the regulation of reverse Ca^{2+} uniport activity (Igbavboa and Pfeiffer, 1991; Litsky and Pfeiffer, 1997) and the Ca^{2+}-activated permeability transition (PT) pore (Bernardi *et al.*, 1992, 1993; Zoratti and Szabo, 1995) of mitochondria. That is why it was interesting to study the effects of divalent metal cations, which can be transported on the Ca^{2+} uniporter, on Cd^{2+}-induced membrane alterations in isolated rat liver mitochondria, and compare their action with the effect of RR, a noncompetitive inhibitor of the Ca^{2+} uniporter (Moore, 1971; Vashington *et al.*, 1972). It is also noteworthy that Cd^{2+} and Ca^{2+} have a similar ion radius, so Cd^{2+} can be used as a probe to study Ca^{2+} action on

ion transport processes and energy metabolism (Skulskii *et al.*, 1991).

MATERIALS AND METHODS

Chemicals

The following chemicals were used: sucrose (refined with help of a cationite KU-2-8 column), KCl, recrystallized twice, and analytical grade $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$, $MnCl_2$, $CdCl_2$, and H_3PO_4 Tris–OH, EGTA, DNP, rotenone, oligomycin, valinomycin, ruthenium red, succinate, ADP, and cyclosporin A were purchased from Sigma Chemical Co (USA); $^{137}Cs^+$ was purchased from Isotop (Russia).

Isolation of Mitochondria

Rat liver mitochondria were isolated from adult male rats (200–250 g) according to Hoogeboom *et al.* (1948), homogenized in a medium containing 250 mM sucrose, 3 mM Tris–HCl, 0.5 mM EGTA (pH 7.4), and resuspended twice in a medium with 250 mM sucrose and 3 mM Tris–HCl (pH 7.2). The protein content was determined according to Lowry *et al.* (1951) and totaled 35–40 mg/ml.

Oxygen Consumption Assay

Respiration (oxygen consumption rate) by mitochondria was monitored at 26°C with a Clark oxygen electrode in 1.2 ml thermostatic closed chamber with magnetic stirring. Mitochondria (2 mg/ml of protein) were added to a medium containing 100 mM KCl, 20 mM Tris–HCl, 3 mM $MgCl_2$, 3 mM Tris–P_i, 5 mM Tris–succinate (pH 7.3). Cd^{2+} (as chloride salt), at tested concentrations, was administered to the medium 1 min prior to the addition of 100 μM ADP in order to induce State 3 (St3) mitochondrial respiration; this procedure was repeated twice. At the end, after the second transition of mitochondria to State 4 (St 4), 32 μM of DNP were administered to the medium (St4 + DNP). Ca^{2+}, Ba^{2+}, Sr^{2+}, and Mn^{2+} were added as chloride salts at 100 μM concentration; the used concentration of ruthenium red was 7.5 μM. Oxygen consumption rates were averaged from five different mitochondrial preparations. Deviations were calculated by the Muller formula for the values and did not exceed 10%.

Mitochondrial Swelling

Cd^{2+} effects on permeability of the inner mitochondrial membrane to K^+ or H^+ were estimated from swelling of nonenergized mitochondria in a medium containing 5 mM Tris–NO_3 (pH 7.4) and 125 mM of KNO_3 or NH_4NO_3, accordingly. Uptake of K^+ by energized mitochondria was determined from their swelling in a medium containing 25 mM K-acetate, 100 mM sucrose, and 10 mM Tris–acetate (pH 7.4). Rotenone (4 μM) and oligomycin (1 μg/ml) were added to all test media up to mitochondrial injection; 5 mM of Tris–succinate was administered where indicated in the figures. Reactions were carried out at 20°C in 2.5-ml chamber filled with an incubation medium and contained 5 mg of mitochondrial protein. The swelling was monitored as apparent absorbance changes at 520 nm with an appropriate recording spectrophotometer (SF-26, LOMO, Russia). The results shown are representative of a series of at least three experiments.

Uptake of ^{137}Cs

Action of Cd^{2+} and Ca^{2+} on uptake of $^{137}Cs^+$ by succinate-energized mitochondria were determined by filtering through 0.4-μM millipore filters (Moore and Pressman, 1964; Skulskii *et al.*, 1983). Kinetics for this process is well characterized by coefficients (r) of distribution of $^{137}Cs^+$ between the energized mitochondria and the medium. These coefficients were calculated from the equation: $r = A_{MT}/A_{MD}$, where A_{MT} is radioactivity in 1 g of mitochondrial protein and A_{MD} is radioactivity in 1 ml of medium. For more details, see Skulskii *et al.* (1983) and the legend to Fig. 8.

RESULTS

Me^{2+} and RR Action on Cd^{2+}-Produced Changes in Respiration of Isolated Mitochondria

As seen from Fig. 1A (trace 1), 5–10 μM Cd^{2+} induced a sustained activation of State 4 respiration of isolated rat liver mitochondria, whereas 12.5–40 μM Cd^{2+} produced its decrease preceded by a transient activation which lasted 15–30 sec (results not shown). Thus, Cd^{2+} action had complex biphasic character and the expression degree of each phase depended on cadmium concentration in the incubation medium. At concentrations of Cd^{2+} exceeding 12.5 μM, the activation phase became shorter and the inhibition started more rapidly. It is noteworthy that data presented here are in a good agreement with those observed in the literature (Sato *et al.*, 1978; Jarvisalo *et al.*, 1980; Sanadi *et al.*, 1981; Cameron *et al.*, 1986).

One can see (Fig. 1A, trace 2) that Cd^{2+} action on State 4 respiration increased considerably after treatment of the mitochondria with 100 μM Ca^{2+}. In this case,

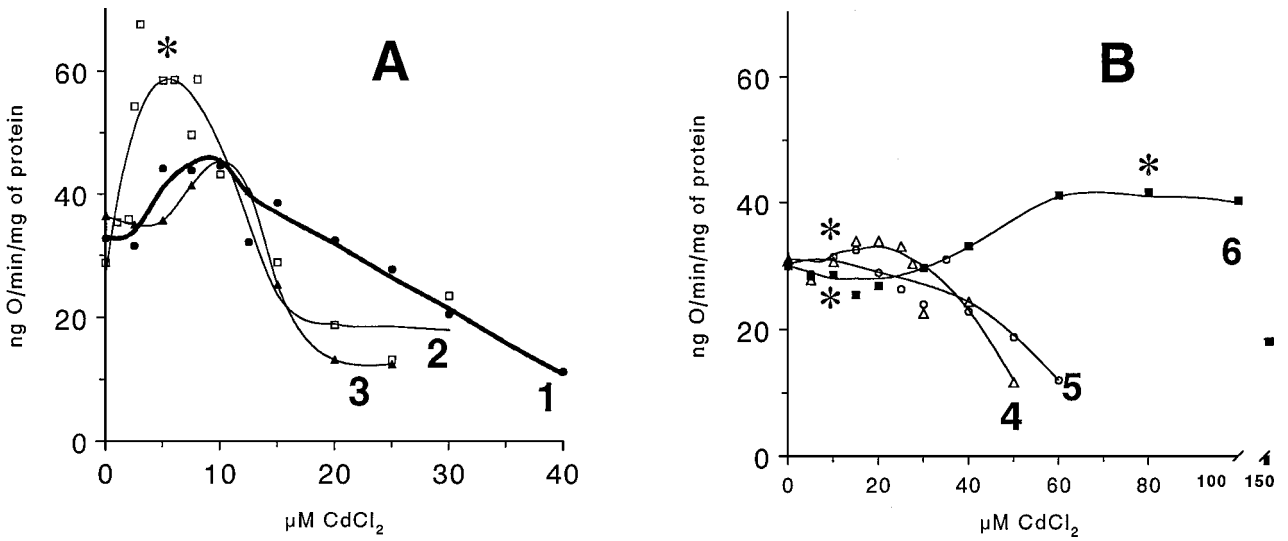

Fig. 1. St4 respiration dependence of isolated mitochondria on concentration of Cd^{2+} in an incubation medium in the presence of Me^{2+} or RR. Rat liver mitochondria (2 mg/ml of protein) were administered to an incubation medium containing 100 mM KCl, 20 mM Tris–HCl, 3 mM $MgCl_2$, 3 mM Tris–P_i, 5 mM Tris–Succ (pH 7.3). This was followed by the additions (A) 1, none (control); 2, 100 μM Ca; 3, 100 μM Ba; (B) 4, 100 μM Sr; 5, 100 μM Mn; 6, 7.5 μM RR. Me^{2+} and RR were presented in the medium from the beginning of experiment. The St4 respiration values performed here are obtained after transition of the mitochondria to resting state following the first addition of ADP (see Materials and Methods). Each point was averaged from five separate mitochondrial preparations yielding reproducible results (±10%). Asterisk indicates statistically significant difference from the mitochondria treated with only $CdCl_2$.

2.5 μM of Cd^{2+} already stimulated State 4 respiration of the mitochondria and did it more significantly than the maximum efficient concentrations of Cd^{2+} (5–10 μM) in control experiments untreated with Me^{2+}; (Fig. 1A, ck, traces 1 and 2). Moreover, in the presence of Ca^{2+}, State 4 respiration was decreased by 15–25 μM of Cd^{2+}, stronger than that in control experiments. After treatment the mitochondria with 100 μM Ba^{2+} (Fig. 1A, trace 3), marginal changes were found in the Cd^{2+} activatory effect on State 4 respiration of these mitochondria in comparison with untreated ones (trace 1); at the same time, inhibition of the respiration produced by cadmium in the range of 12.5–25 μM was more severe and, in this case, resembled that observed in the presence of Ca^{2+} (trace 2). The effects of Cd^{2+} on State 4 respiration markedly decreased in experiments with mitochondria treated with 100 μM of Sr^{2+} (Fig. 1B, trace 4) or Mn^{2+} (Fig. 1B, trace 5). Sr^{2+}-treated mitochondria showed only a slight stimulation of State 4 respiration by cadmium, after transition of the mitochondria to the resting state following the first addition of ADP (Fig. 1B, trace 4), while there was not any stimulation of the respiration in the resting state after the second ADP addition (Table I). This stimulation was not found at all in experiments with Mn^{2+}-treated mitochondria (Fig. 1B, trace 5; Table I). In turn, the inhibition of State 4 respiration of Sr^{2+}-or Mn^{2+}-treated mitochondria induced by cadmium became substantial only when Cd^{2+} concentrations in the medium exceeded 40 μM. The marked changes in Cd^{2+} action on State 4 respiration of the mitochondria were observed in the presence of 7.5 μM RR in an incubation medium (Fig. 1B, trace 6). In this case, the stimulation

Table I. Changes in Stimulatory Action of Cd^{2+} on State 4 Respiration of Isolated Rat Liver Mitochondria Affected by Divalent Cations or Ruthenium Red[a]

Additions	Activation (in % to corresponding control) St4 (1)	St4 (2)	Range of maximum efficient concentrations of Cd^{2+} (μM)
Cd	18 ± 0.7	29 ± 1.1	5–10
Cd + Ca	135 ± 12	142 ± 16	2.5–7.5
Cd + Ba	24 ± 1.4	31 ± 2.8	7.5–12.5
Cd + Sr	19 ± 1.3	9 ± 0.5	15
Cd + Mn	11 ± 0.8	7 ± 0.5	15
Cd + RR	53 ± 2.7	38 ± 1.9	60–100

[a] Ca^{2+}, Ba^{2+}, Sr^{2+} or Mn^{2+} were added as chloride salts in concentration of 100 μM; concentration of RR was 7.5 μM. In each case, oxygen consumption rate values in the absence of cadmium chloride in an incubation medium (controls) were compared with those in the presence of maximum effective concentrations of Cd^{2+} (at given experimental conditions). Numbers in parentheses indicate that presented St4 respiration rate values were measured after the transition of mitochondria to resting state following the first (1) or second (2) addition of ADP (see Materials and Methods). Mean values ± SD for five different mitochondrial preparations are shown.

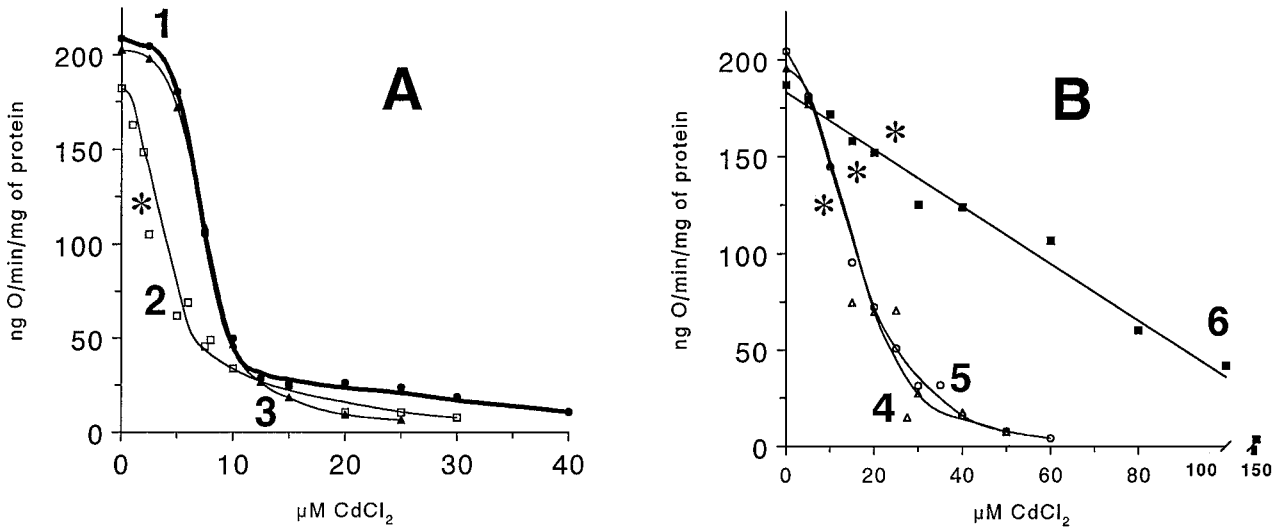

Fig. 2. DNP-stimulated (St4 + DNP) respiration dependence of isolated mitochondria on concentration of Cd^{2+} in an incubation medium in the presence of Me^{2+} or RR. Each point was averaged from five separate mitochondrial preparations yielding reproducible results (±6%). Asterisk indicates statistically significant difference from the mitochondria treated with $CdCl_2$ only. Designations, concentration of the mitochondria, and incubation medium are as in Fig. 1.

of State 4 respiration by cadmium chloride occurred only at Cd^{2+} concentrations exceeding 40 μM and it was observed up to 100 μM Cd^{2+} in the medium. It is also seen that, in the presence of RR, State 4 respiration became depressed only in the range 100–150 μM of cadmium, reaching significant inhibition at 150 μM of Cd^{2+} in the medium.

Therefore, the evidence found here revealed that the final activation (see Table I) of State 4 respiration induced by cadmium at its maximum efficient concentrations (5–10 μM) in control mitochondria (untreated with Me^{2+}) was close to 30%. At the same time, for Ca^{2+}-treated mitochondria, the State 4 respiration rate values in the presence of the most efficient cadmium concentrations at given experimental conditions (2.5–7.5 μM) were more than two times higher than in the absence of Cd^{2+}; moreover, the activation phase started at two times lower concentrations of Cd^{2+} as compared with controls. In the presence of 100 μM of Sr^{2+} or Mn^{2+} in the incubation medium, any stimulation (if so) of State 4 respiration was observed only at 15 μM of Cd^{2+}. However, the final activation, in these cases (see Table I), was marginal (less than 10%). When 7.5 μM RR was present in the medium, the activation of the basal respiration was obtained only at 60 μM of Cd^{2+} and was close to 40% (Table I). It is necessary to point out that Ba^{2+} itself, at tested concentrations (100 μM), did not influence the oxygen consumption rate by the mitochondria in State 4, while all other Me^{2+} under study at this concentration, as well as 7.5 μM of RR used per se, decreased State 4 respiration by 15–25%. Similar data concerning the inhibitory action of RR and Me^{2+} (used at close concentrations) on basal respiration of isolated rat liver mitochondria were discussed in the literature (Vashington *et al.*, 1972; Diwan, 1985).

As to Cd^{2+} action on an uncoupler-stimulated respiration, we have shown (Fig. 2A, trace 1) that 5–10 μM Cd^{2+} substantially decreased DNP-stimulated respiration of isolated rat liver mitochondria. Starting from 12.5 μM Cd^{2+}, DNP addition had no effect on the mitochondrial respiration, so an almost complete inhibition of oxygen consumption by the mitochondria was achieved by 12.5 μM cadmium. The IC_{50} value (concentration required to inhibit the maximal response by 50%) for Cd^{2+} was found to be 7.5 μM. Minimal and essentially maximal inhibition of the uncoupler-stimulated respiration was observed within a narrow concentration range for cadmium chloride (5–10 μM), so approximately a twofold increase in cadmium concentration in the incubation medium produced these drastic changes (Fig. 2A). All this coincides well with data obtained previously on FCCP-uncoupled rat liver mitochondria affected by cadmium chloride (Miccadei and Floridi, 1993).

In experiments with Ca^{2+}-treated mitochondria (Fig. 2A, trace 2), the uncoupler-stimulated respiration of the mitochondria affected by cadmium was strongly inhibited and this effect was observed from lower cadmium concentrations than those in control experiments (untreated with Me^{2+}). The IC_{50} value for cadmium chloride in the presence of Ca^{2+} was equal to 2.5 μM. As for Ba^{2+}-treated mitochondria (Fig. 2A, trace 3), on the one hand,

the action of Cd^{2+} in the range 2.5–10 μM was consistant with its action in controls. On the other hand, 12.5–25 μM of Cd^{2+} inhibited the uncoupler-stimulated respiration of Ba^{2+}-treated mitochondria in the same way as occurred in experiments with Ca^{2+}-treated mitochondria. The IC_{50} value for cadmium chloride in the presence of Ba^{2+} was found to be 7.5 μM. The Cd^{2+} action on DNP-stimulated respiration of Sr^{2+}- (Fig. 2B, trace 4) or Mn^{2+}-treated (Fig. 2B, trace 5) mitochondria was significantly changed in comparison with controls. The IC_{50} value for cadmium chloride, in both cases, was close to 15 μM. In the presence of RR (7.5 μM) in the incubation medium (Fig. 2B, trace 6), the uncoupler-stimulated respiration decreased slowly with a rise of cadmium chloride concentrations from 5 to 150 μM, where a maximum decrease of the mitochondrial respiration was observed. This relationship has a linear mode with the correlation coefficient being equal 0.986 ($p < 0.001$; the STATGRAPHICS program, version 3). The IC_{50} value for cadmium chloride in the presence of RR was equal to 60 μM. It should be mentioned that RR and all Me^{2+} examined (except Ca^{2+}) did not affect the uncoupler-stimulated respiration per se (at tested concentrations), whereas 100 μM Ca^{2+} decreased DNP-stimulated respiration by 13% (see Fig. 2).

As shown in Fig. 3, ADP-stimulated respiration of isolated rat liver mitochondria was also depressed by the addition of cadmium chloride in the incubation medium. In experiments with control mitochondria, Cd^{2+} (2.5–12.5 μM) decreased State 3 respiration of mitochondria (Fig. 3A, trace 1) and maximum inhibition, followed by the complete disappearance of susceptibility of the mitochondria to ADP addition, was observed at 12.5 μM of Cd^{2+}, achieving an average of 53%. At concentrations of Cd^{2+} exceeding 12.5 μM (not shown here), there was no response of the mitochondria to any additions of ADP, so the respiratory control index (RCI) was equal to 1. On the whole, data obtained by us here are in good agreement with previously found on isolated rat liver (Sato *et al.*, 1978) and heart (Kisling *et al.*, 1987) mitochondria.

It is seen also that State 3 respiration of Ca^{2+}-treated mitochondria (Fig. 3A, trace 2) affected by cadmium chloride was inhibited more strongly in comparison with that of controls (untreated with Me^{2+}). In this case, the reaction of the mitochondria to the second addition of ADP was already not observed at 7.5 μM Cd^{2+} in the incubation medium. The effect of Cd^{2+} on State 3 respiration of Ba^{2+}-treated mitochondria (Fig. 3A, trace 3) remained practically the same as that found for control, except that ADP-stimulated respiration slightly, but significantly, had already decreased in the presence of 100 μM of barium chloride alone (without any cadmium chloride) in the incubation medium. As to Sr^{2+}-(Fig. 3B, trace 4) or Mn^{2+}-treated (Fig. 3B, trace 5) mitochondria, their State 3 respiration became "more tolerant" to a rise of Cd^{2+} concentrations in the medium in comparison with that of untreated mitochondria. State 3 respiration of these mitochondria was not changed significantly by the addition of 5 to 15 μM of Cd^{2+}. Thus, the protective action of these

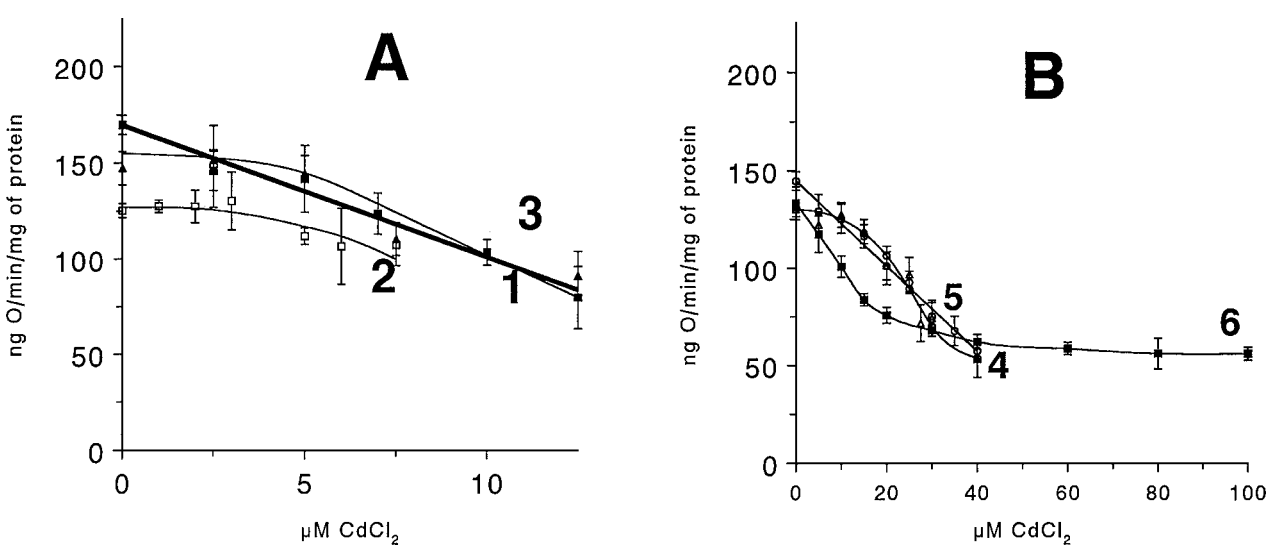

Fig. 3. St3 respiration dependence of isolated mitochondria on concentration of Cd^{2+} in an incubation medium in the presence of Me^{2+} or RR. State 3 respiration values shown are obtained after the first addition of ADP to the incubation medium (see Materials and Methods) and are presented here up to that Cd^{2+} concentration starting from which the response of the mitochondria on the second ADP addition has not already been observed. Statistics and the remainder are as in Fig. 1, except that here mean values ± SD.

divalent cations is really evident. Marked decline of the mitochondrial respiration in the presence of Sr^{2+} or Mn^{2+} in the incubation medium was found only at 20–40 μM of Cd^{2+} (Fig. 3B, traces 4 and 5); in turn, when the concentrations of cadmium chloride exceeding 40 μM (not shown here), there was no response of the mitochondria at all upon addition of ADP. On the contrary, in the presence of 7.5 μM RR in the incubation medium (Fig. 3B, trace 6), ADP-stimulated respiration was already substantially depressed by 5–10 μM of Cd^{2+}; moreover, the inhibition seems to be even more pronounced than that observed in the case of control mitochondria (Fig. 3A, trace 1). As also seen, State 3 respiration of RR-treated mitochondria was inhibited in the presence of cadmium chloride significantly more than that of Sr^{2+}-or Mn^{2+}-treated mitochondria (Fig. 3B, trace 4 and 5) up to 30 μM of Cd^{2+} in the incubation medium. One can see that in the presence of RR, there is a linear relationship between oxygen consumption rate values and cadmium chloride concentrations up to 15 μM of Cd^{2+} in the medium, with a correlation coefficient being equal to 0.999 ($p < 0.001$). After that, there is a plateau of the dependence up to 100 μM of Cd^{2+} in the incubation medium. Therefore, RR-treated mitochondria were susceptible to the addition of ADP even at 40–100 μM Cd^{2+} in the reaction medium (Fig. 3B, trace 6); however, they also lost their sensibility to ADP addition at the concentrations of cadmium chloride exceeding 100 μM (not presented here).

In conclusion, it is noteworthy that 100 μM of all Me^{2+} under investigation per se inhibited oxygen consumption by the mitochondria in State 3 respiration. The least inhibitory effect was obtained in the case of Ba^{2+} and was equal to 11–13%; in the presence of Sr^{2+} or Mn^{2+} in the incubation medium, a decrease in State 3 respiration was found to be 17–23 and 15–20%, respectively. The most inhibitory effect among Me^{2+} tested was obtained in Ca^{2+}-treated mitochondria, which exceeded 25%. RR at the used concentration (7.5 μM) also decreased State 3 respiration of the mitochondria by 15–21%. Analogous studies of the effects of RR and Me^{2+} on State 3 respiration of isolated rat liver mitochondria (at close concentrations) was observed previously by others (Caplan and Carafoli, 1965; Vashington *et al.*, 1972; Diwan, 1985; Moreno-Sanchez, 1985; Gavin *et al.*, 1992).

Me^{2+} and RR Action on Cd^{2+}-Induced Ion Permeability of the Inner Membrane in Isolated Mitochondria

In Cd^{2+}-free experiments, nonenergized rat liver mitochondria swelled very slowly in a medium containing 125 mM NH_4NO_3. Treatment of the mitochondria with 100 μM Ca^{2+}, Ba^{2+} or Sr^{2+} did not affect the swelling of these mitochondria in NH_4NO_3 medium (results not shown). In turn, the swelling of nonenergized mitochondria treated with 100 μM Mn^{2+} or 7.5 μM RR was slightly enhanced in comparison with controls (untreated with Me^{2+}) (data not performed). The energization of control mitochondria by succinate (5 mM) resulted in the arrest of swelling of mitochondrial suspension 1 min following after its addition. The absorbance of Me^{2+}- or RR-treated mitochondria was stabilized by succinate addition in a similar manner (not demonstrated here).

Swelling of nonenergized mitochondria incubated in NH_4NO_3 medium was stimulated slightly (but significantly) by 10 μM Cd^{2+} (Fig. 4A, trace 1), while 5 μM Cd^{2+} did not stimulate this swelling at all (data not presented). Conversely, 15 and, especially, 20 μM of Cd^{2+} showed extremely high-amplitude swelling of the mitochondria in this medium (not performed here). Thus, in NH_4NO_3 medium, cadmium chloride induced the swelling of nonenergized mitochondria in a dose-dependent manner.

Swelling in NH_4NO_3 medium of nonenergized mitochondria treated with 100 μM Ba^{2+}, Mn^{2+}, Sr^{2+} or 7.5 μM RR (Fig. 4A, traces 3–6, respectively) in the presence of 10 μM cadmium chloride in the incubation medium was approximately the same as that of controls (Fig. 4A, trace 1). Conversely, the swelling of Ca^{2+}-treated mitochondria in the presence of 10 μM Cd^{2+} increased substantially in this medium (Fig. 4A, trace 2). In turn, energization of control mitochondria by the addition of succinate (5 mM) markedly stimulated the swelling in NH_4NO_3 medium (Fig. 4A, trace 1). Likewise, the swelling of Ca^{2+}-treated mitochondria affected by 10 μM cadmium chloride was stimulated, in addition, by succinate (Fig. 4A, trace 2). On the other hand, in the presence of 10 μM of cadmium chloride in the incubation medium, the swelling of isolated mitochondria treated with Ba^{2+}, Mn^{2+}, Sr^{2+} or RR was inhibited after the succinate addition with the selectivity series RR > Sr^{2+} > Mn^{2+} > Ba^{2+} (see Fig. 4A, traces 3–6, respectively).

A similar picture was obtained in NH_4NO_3 medium for energized mitochondria when succinate was added in the buffered medium from the start of experiment (Fig. 4B). In this case, Me^{2+} (Ca^{2+}, Ba^{2+}, Mn^{2+} or Sr^{2+}) were administered 1 min after the addition of the mitochondria and cadmium (Fig. 4B, traces 2–5, respectively). It is seen that, as compared to the control mitochondria with 15 μM Cd^{2+} alone presented in the incubation medium (Fig. 4B, trace 1), 100 μM of Ba^{2+}, Mn^{2+} or Sr^{2+} produced shrinkage of the mitochondrial suspension in NH_4NO_3 medium, whereas 100 μM of added Ca^{2+} enhanced Cd^{2+}-induced swelling in this medium (Fig. 4B,

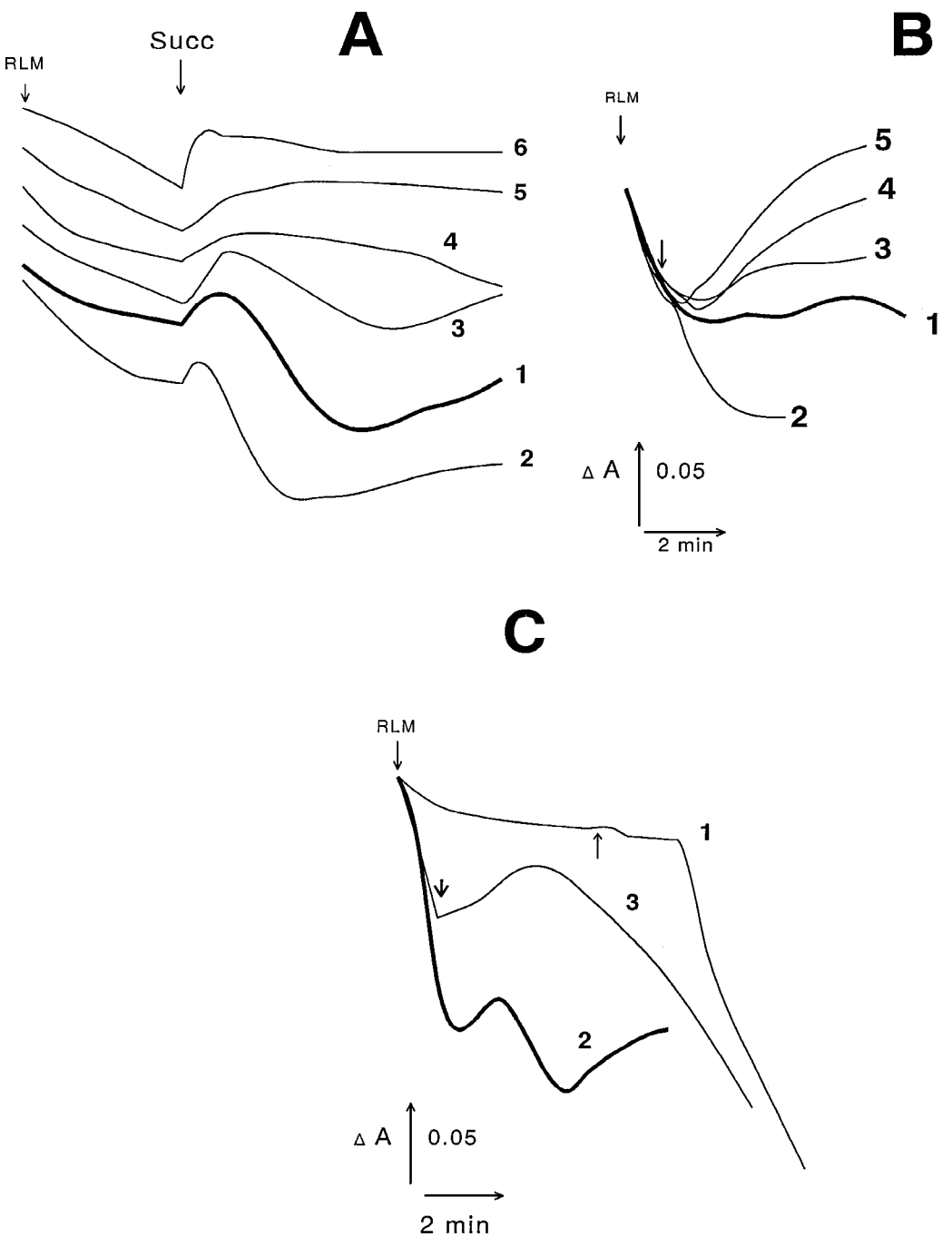

Fig. 4. Effects of Cd^{2+} on swelling of isolated mitochondria in 125 mM NH_4NO_3 medium in the presence of Me^{2+} or RR. Rat liver mitochondria (2 mg/ml of protein) were administered to a reaction medium (see Materials and Methods) containing the following: (A), 10 μM Cd; (B and C), 15 μM Cd. The other additions in panels (A) and (B) were: 1, none; 2, 100 μM Ca; 3, 100 μM Ba; 4, 100 μM Mn; 5, 100 μM Sr; 6, 7.5 μM RR. The additions used for panel (C) were: 1, none; 2 and 3, Cd in the absence (2) or presence (3) of RR and Ca. In panel (A), Cd^{2+}, Me^{2+} or RR were administered to the medium before the mitochondria. Succinate was added where indicated. In panels (B) and (C), the additions of Me^{2+}(B) or 7.5 μM RR and 100 μM Ca (C) were made 1 min after the mitochondria and cadmium; succinate, in these cases, was presented in the incubation medium from the start of the experiment. Me^{2+}(↓), RR + Ca(↓, bold) or DNP(↑) were added where indicated. Typical traces for four separate mitochondrial preparations are shown. For the remainder, see Materials and Methods.

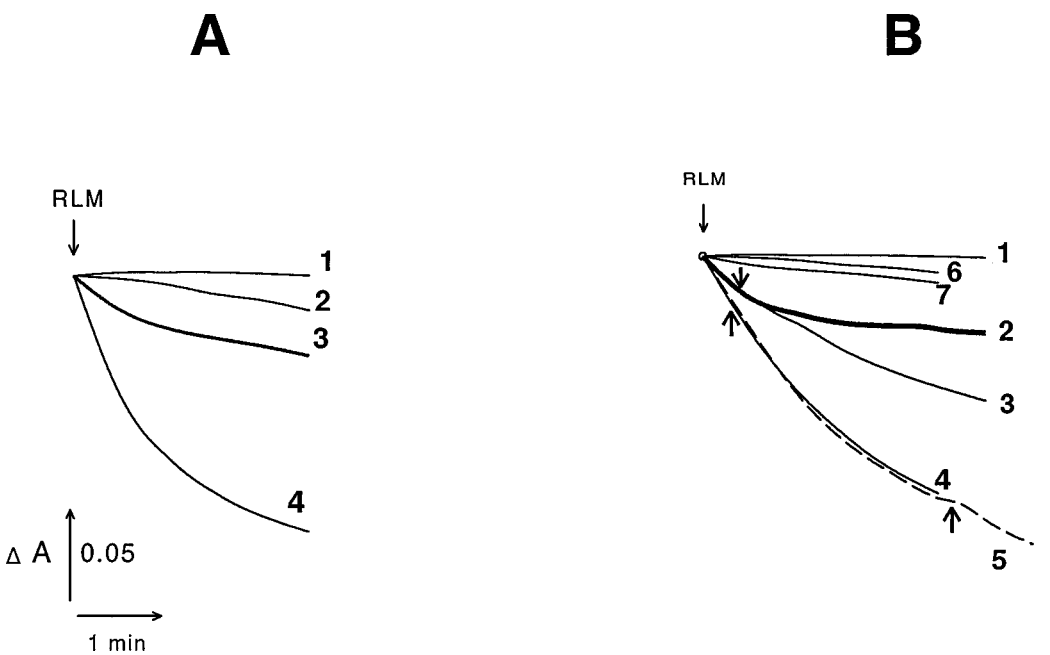

Fig. 5. Effects of Cd^{2+} on swelling of nonenergized mitochondria in 125 mM KNO_3 medium in the presence of Me^{2+} or RR. The following additions were made: (A) 1, none; 2–4, 10 μM Cd alone (3) or together with RR (2) or Ca (4); (B) 1, none; 2–7, 10 μM Cd; 3, RR and Ca were simultaneously administered 1 min after the mitochondria and cadmium; 4 and 5, Ca^{2+} was presented in the medium from the beginning of the experiment, while RR was added 1 (4) or 6 min (5) after the mitochondria and cadmium; 6 and 7, RR was contained in the medium from the start of incubation in the absence (6) or in the presence (7) of Ca^{2+}. RR + Ca (↓), RR (↑) were added where indicated by arrows. The concentrations of the mitochondria, Me^{2+} and RR used are as in Fig. 4. Typical traces for three different mitochondrial preparations are presented. For all other details, see Materials and Methods.

trace 2). It is also interesting that the addition to the incubation medium of 100 μM Ca^{2+} simultaneously with RR (7.5 μM) 1 min after the mitochondria and cadmium were added produced additional enhancement of swelling of energized mitochondria in NH_4NO_3 medium (Fig. 4C, trace 3; compare with trace 2, control).

It was observed, as before, that nonenergized rat liver mitochondria did not swell in KNO_3 medium (Fig. 5A, trace 1). In the case of Ca^{2+}-, Ba^{2+}-, Sr^{2+}-, Mn^{2+}-, or RR-treated mitochondria, no swelling occurred, in Cd^{2+}-free experiments (unpresented data). However, we found slight, but significant, stimulation of swelling of untreated Me^{2+} mitochondria in KNO_3 medium in the presence of 10 μM Cd^{2+} (Fig. 5A, trace 3). This stimulating effect of 10 μM Cd^{2+} was not changed after treatment of mitochondria by 100 μM Ba^{2+}, Sr^{2+} or Mn^{2+} (results not performed), but decreased markedly in the presence of 7.5 μM RR in the incubation medium (Fig. 5A, trace 2). On the contrary, Ca^{2+}-treated mitochondria demonstrated substantial enhancing of swelling in KNO_3 medium in the presence of 10 μM cadmium chloride (Fig. 5A, trace 4). Moreover, as demonstrated in Fig. 5B, 100 μM Ca^{2+} simultaneously administered with RR in the incubation medium 1 min after the mitochondria and cadmium enhanced the swelling of nonenergized mitochondria in KNO_3 medium in an additional way (trace 3, compare with trace 2, control). It is seen that the addition of RR in the medium containing both Ca^{2+} (100 μM) and Cd^{2+} (10 μM) 1 (trace 4) or 6 min (trace 5) after treatment had no effect on the swelling of the mitochondria, while its addition in the incubation medium simultaneously with 10 μM of cadmium in the absence (trace 6) or presence (trace 7) of 100 μM Ca^{2+} completely halted the swelling of the mitochondrial suspension in KNO_3 medium. It is note-worthy that 5 μM Cd^{2+} did not produce any swelling in KNO_3 medium, while 15 μM and, in particular, 20 μM of cadmium chloride induced large-amplitude swelling in this medium (data not shown). Therefore, Cd^{2+}-induced swelling of nonenergized mitochondria in KNO_3 medium, as in NH_4NO_3 medium (see above), was dose dependent.

As presented in Fig. 6, nonenergized rat liver mitochondria did not swell in a medium containing K-acetate (25 mM) and sucrose (100 mM). This statement is not only valid for experiments with control mitochondria (Fig. 6, panels A and B, trace 1), but also for experiments

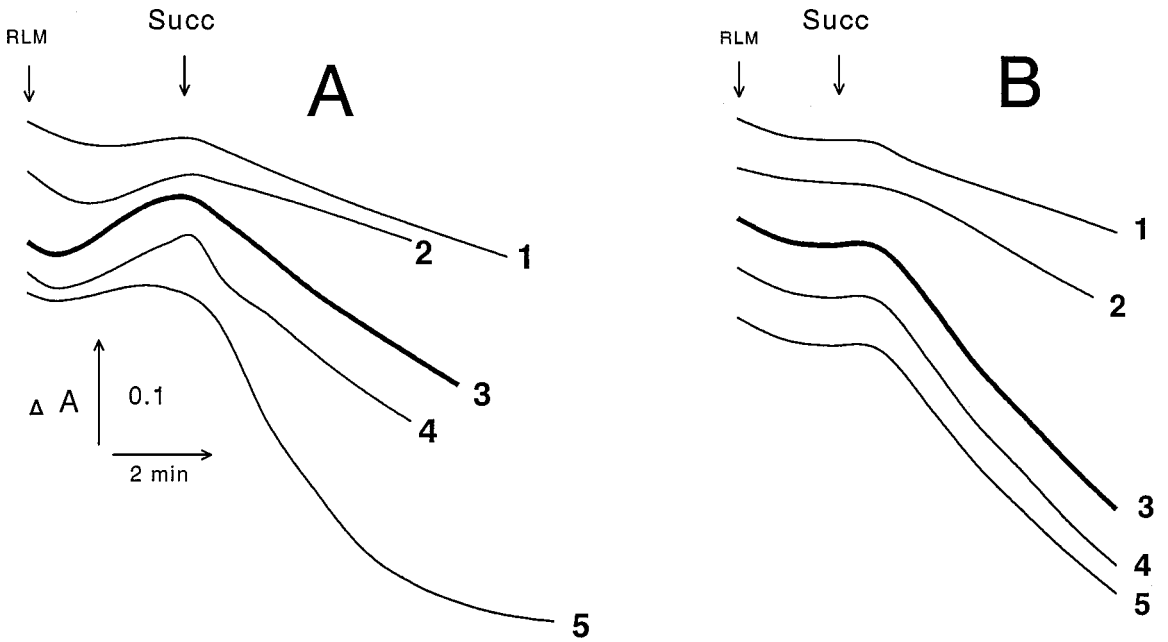

Fig. 6. Effects of Cd^{2+} on swelling of isolated mitochondria in K-acetate medium (25 mM K acetate and 100 mM sucrose) in the presence of Me^{2+} or RR. The following additions were made: 1, none (A;B); 2–5, in the absence (A) or in the presence (B) of RR; 2, 100 μM Me^{2+} (A) or 7.5 μM RR (B); 3, 10 μM Cd; 4, 10 μM Cd and 100 μM Me^{2+} (except Ca); 5, 10 μM Cd and 100 μM Ca. Succinate was administered where indicated. The concentration of the mitochondria and statistics are as in Fig. 4. For details, see Materials and Methods.

with Me^{2+}- (Fig. 6A, trace 2) or RR-treated (Fig. 6B, trace 2) ones. As seen, energization of control mitochondria by succinate (5 mM) markedly stimulated mitochondrial swelling. In experiments with Me^{2+}- or RR-treated mitochondria, the swelling of mitochondrial suspension, after the addition of succinate, also occurred and was not changed significantly, as compared with untreated ones.

Swelling of control mitochondria in the above-mentioned K-acetate medium, after their energization by succinate, was also stimulated by 10 μM Cd^{2+} (Fig. 6A, trace 3). Swelling of Ba^{2+}-, Sr^{2+}- or Mn^{2+}-treated mitochondria, affected by 10 μM cadmium chloride and energized by succinate a few minutes later, was the same as in the case of controls (Fig. 6A, trace 4; compare with trace 3). Conversely, the swelling of Ca^{2+}-treated mitochondria in the presence of 10 μM Cd^{2+} was substantially enhanced after succinate addition, compared to mitochondria treated with the rest Me^{2+} under test conditions. (Fig. 6A, trace 5; compare with trace 4).

It is important that in the presence of RR in the medium, the activation of mitochondrial swelling by 10 μM of cadmium chloride after the addition of succinate also occurred (Fig. 6B, trace 3). It was even more significant than that under the same conditions, but in the absence of RR (Fig. 6A, trace 3). These data coincide well with those obtained earlier by others (Rasheed *et al.*, 1984). Moreover, in this case, there was no additional stimulation of the swelling by 100 μM Ca^{2+} (Fig. 6B, trace 5) and the swelling of mitochondrial suspension in the presence of Ca^{2+} was the same as in the presence of other divalent cations under study (Fig. 6B, trace 4).

Remarkably, in contrast with the situation observed in KNO_3 and NH_4NO_3 media (see above), 5 μM of cadmium chloride (data not shown) already induced the swelling in K-acetate medium in the presence of RR. In this case, the following rise of Cd^{2+} concentration in the buffered medium to 15–20 μM increased the swelling of the mitochondrial suspension in a dose-dependent manner (results not presented). In the absence of RR, on the contrary, the further increase of cadmium concentration up to 20 μM (unperformed evidence) depressed Cd^{2+}-induced swelling in K-acetate medium in comparison with the mitochondrial swelling produced by 10 μM of Cd^{2+} (Fig. 6A, trace 3). Moreover, in the initial period of the incubation (10 min), the swelling at given experimental conditions was even less than in control mitochondria in the absence of cadmium (Fig. 6A, trace 1).

To elucidate the mechanism of Cd^{2+}-induced permeability of the inner mitochondrial membrane to K^+ and

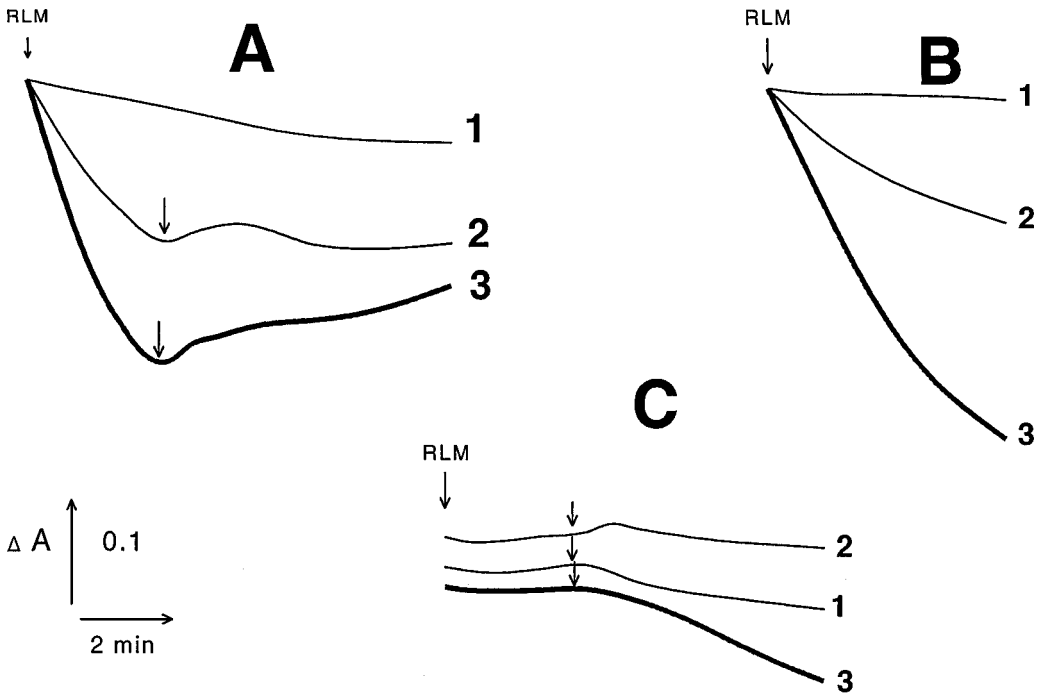

Fig. 7. Action of cyclosporin A on Cd^{2+}-induced swelling in NH_4NO_3 (A), KNO_3 (B), and K-acetate (C) media. The following additions were used: 1, 1 μM CSA (control); 2, 15 μM Cd and 1 μM CSA; 3, 15 μM Cd. CSA and Cd^{2+} were added prior to the mitochondria. Succinate was administered where indicated by arrows. The concentrations of the mitochondria and statistics are as in Fig. 4. For all other details, see Materials and Methods.

H^+, we investigated the action of cyclosporin A (CSA), a high-potency inhibitor of mitochondrial permeability transition pore (Zoratti and Szabo, 1995), on Cd^{2+}-produced swelling in all media under test. We determined that, in all cases, mitochondrial swelling activated by 15 μM of Cd^{2+} was inhibited completely or partially by 1 μM CSA presented in an incubation medium from the start of experiment (Fig. 7).

In conclusion, we have examined Cd^{2+} effects on uptake of $^{137}Cs^+$ (as an analog of K^+) by succinate-energized rat liver mitochondria using a millipore filtration method (see Materials and Methods). As can be seen from Fig. 8A, at 20°C, 5 μM Cd^{2+} in the presence of RR (trace 4) already induced the marked increase of $^{137}Cs^+$ uptake rate in acetate medium (50 mM Tris-acetate and 140 mM sucrose) in comparison with control (trace 2). This coincide well with our swelling data discussed above. An analogous effect, but of lesser magnitude, was obtained at 0°C (Fig. 8A, traces 1 and 3). In addition, the uptake of $^{137}Cs^+$ by energized mitochondria in the presence of valinomycin, estimated by *r* coefficient (all details in Materials and Methods), characterizes, as is well known, the energetic condition of isolated mitochondria and, indirectly, the mitochondrial membrane potential ($\Delta\psi_{mito}$) value. In our experiments, the addition of 10 μM Cd^{2+} at 20°C in the acetate medium containing 10^{-8} μM of valinomycin resulted in outflow of $^{137}Cs^+$ from the mitochondrial matrix that was established by the sharp decline of the r_{Cs^+} coefficient values (Fig. 8B, trace 1; see also Korotkov *et al.*, 1999). A lesser effect, but still considerable, was found in the presence of 5 μM cadmium chloride in the medium (unpresented data), while 2 μM Cd^{2+} had only the weak action (Fig. 8B, trace 3). Bivalent metal ions (Ba^{2+}, Sr^{2+}, Mn^{2+}) at concentrations of 10 μM in the incubation medium did not affect the r_{Cs^+} coefficient values (results not shown). In turn, 10 μM Ca^{2+} slightly decreased the uptake of $^{137}Cs^+$, although its effect was marginal (Fig. 8B, trace 4). More substantially, Ca^{2+} (10 μM) enhanced the effect of Cd^{2+} (2 μM) when simultaneously added to the incubation medium (Fig. 8B, trace 2).

We have previously found (Korotkov *et al.*, 1999) that at 20°C RR antagonizes the process induced by cadmium addition. The effects of RR and Cd^{2+} at 0°C (Fig. 8C) were the same, with the exception that there was slower decrease of r_{Cs^+} values, as well as Cd^{2+} effects, produced by cadmium chloride at this temperature (Fig. 8C, trace 1), depended very little on its concentration in the incubation medium (evidence not presented).

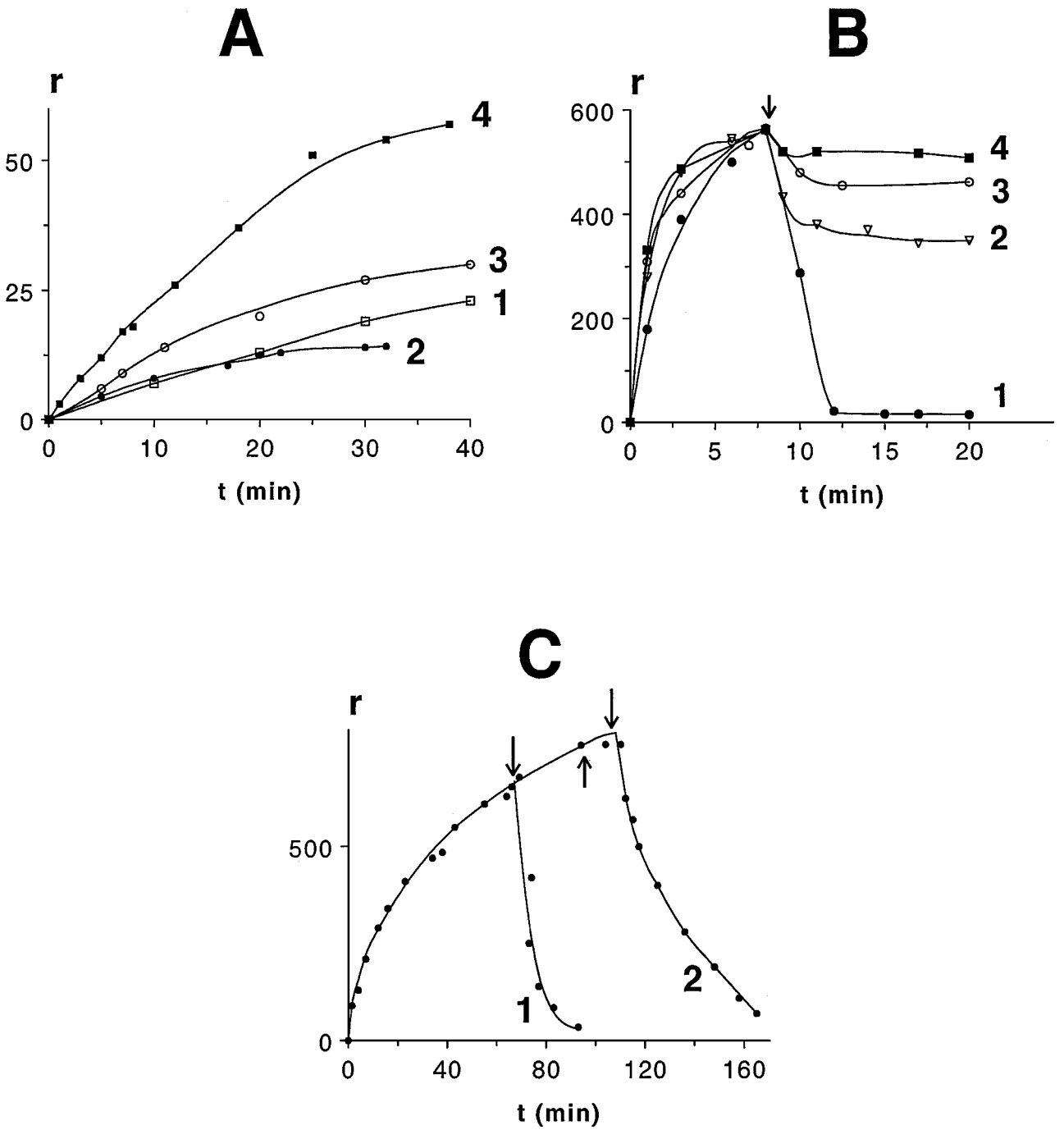

Fig. 8. Effects of Cd^{2+}, Ca^{2+}, and RR on the distribution of $^{137}Cs^{+}$ between isolated succinate-energized mitochondria and an incubation medium. Rat liver mitochondria (1 mg/ml of protein) were administered at 0°C (A, traces 1 and 3; C) and 20°C (A, traces 2 and 4; B) to a medium containing 140 mM sucrose and 50 mM Tris–acetate (pH 7.4), 2.5 mM of Tris–succinate, 5 μM of rotenone, 1 mg/ml of oligomycin in the absence (A) or in the presence (B;C) of valinomycin (10^{-8} M). The following additions were made (A) in the presence of 7.5 μM RR: 1 and 2, none; 3 and 4, 5 μM Cd (administered before the mitochondria); (B) 1, 10 μM Cd; 2, 2 μM Cd and 10 μM Ca; 3, 2 μM Cd; 4, 10 μM Ca; (C)1, 15 μM Cd; 2, 15 μM Cd after RR (7.5 μM). During the long-lasting incubation, when the mitochondria reached their steady states at given experimental conditions, Cd^{2+} (B,C), Me^{2+} (B), or RR (C) were administered where indicated by arrows (↑, RR; ↓, Cd^{2+} or/and Me^{2+}). Typical traces for four (A), two (B), and three (C) separate mitochondrial preparations are presented.

DISCUSSION

In this paper we have presented the results of a detailed study to determine effects of cadmium (0–150 μM) on oxygen consumption by isolated rat liver mitochondria in the following states of respiration: State 4 (basal, coupled, no ADP), DNP-stimulated (uncoupled), and State 3 (coupled, phosphorylating with maximum ADP). We revisited the long-known effects of Cd^{2+} on mitochondrial respiration, looking at how dose dependence was modified by treatment with divalent metal ions (Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+}) or ruthenium red, correspondingly competitive and noncompetitive inhibitors of the Ca^{2+} uniporter of the inner mitochondrial membrane, which is generally considered to be responsible for Cd^{2+} transport into the matrix (Saris and Jarvisalo, 1977; Jarvisalo *et al.*, 1980; Rasheed *et al.*, 1984; Chavez *et al.*, 1985). The most significant findings are following:

i. We have confirmed that Cd^{2+} has a biphasic action on State 4 respiration, inducing a sustained activation in the range of 5 to 10 μM of cadmium in KCl medium and a transient activation followed by the return to basal level or inhibition at Cd^{2+} concentrations exceeding 12.5 μM. More importantly, we have shown (Fig. 1) that Ca^{2+} enhances the stimulatory effect of Cd^{2+} more than two times and shifts its maximum effective concentrations from 5–10 μM to 2.5–7.5 μM of cadmium chloride in the medium. Mn^{2+} and Sr^{2+}, in contrast, inhibit the activation effect of Cd^{2+}, and a marginal stimulation by Cd^{2+} in their presence is obtained (if so) only at 15 μM of cadmium in the buffered medium (Table I). The activation of State 4 respiration by Cd^{2+} in the presence of RR in the incubation medium still takes place but is observed at Cd^{2+} concentrations more than one order higher (60–100 μM) than in the absence of RR (Table I).

ii. The uncoupler-stimulated respiration, on the contrary, is inhibited by cadmium chloride starting from 2.5 μM Cd^{2+}, a finding previously obtained by us (Skul'skii *et al.*, 1988) and others (Miccadei and Floridi, 1993). In the case of FCCP, the IC_{50} value for Cd^{2+} was found originally to be 4.7 μM (Miccadei and Floridi, 1993) and, in our case (with DNP as uncoupler), the IC_{50} value for cadmium chloride was 7.5 μM. More significantly, data presented here (Fig. 2) show that Ca^{2+} decreases the value of the IC_{50} for cadmium chloride (the IC_{50} was found to be 2.5 μM in the presence of Ca^{2+}), while Sr^{2+}, Mn^{2+}, and especially RR increase the IC_{50} value of Cd^{2+} (the IC_{50} was equal to 15 μM in the presence of Sr^{2+} or Mn^{2+} and 60 μM in the presence of RR). In agreement with this, there is information in the literature that all divalent cations under study induce oxidation of cytochrome *b* (Vainio *et al.*, 1970) including Cd^{2+} (Sato *et al.*, 1978; Miccadei and Floridi, 1993). It also seems important that in all these cases the sustained activation of State 4 respiration induced by cadmium is observed starting from those Cd^{2+} concentrations, at which a half-maximal inhibition of the uncoupler-stimulated respiration produced by Cd^{2+} has been already achieved or, at least, from the concentrations extremely close to IC_{50} values for cadmium in a given set of experimental conditions.

iii. ADP-stimulated respiration, as shown by some workers (including us), is also depressed by cadmium at concentrations exceeding 2–3 μM (Sato *et al.*, 1978; Cameron *et al.*, 1986; Skul'skii *et al.*, 1988). Here, we have found that in our experimental model (see Materials and Methods), the response of control mitochondria upon the second addition of ADP disappears at 12.5 μM Cd^{2+} in the medium (Fig. 3). Moreover, in the presence of Ca^{2+}, this effect is observed at lower Cd^{2+} concentrations (7.5 μM), whereas in the presence of Sr^{2+} (Mn^{2+}) or RR it is obtained at higher concentrations of Cd^{2+} (40 and 100 μM, correspondingly). It is note worthy, at the same time, that at given concentrations of Cd^{2+}, State 3 respiration is inhibited only by 14% (in the presence of Ca^{2+}) and 53% (in the case of control mitochondria), while it decreases 60, 59, and 58% in the presence of Mn^{2+}, Sr^{2+} or RR, respectively. It is also substantial in our opinion, that only at the concentrations of Cd^{2+} exceeding the ones given (namely, 7.5 μM for Ca^{2+}, 12.5 μM for control, 40 μM for Sr^{2+} or Mn^{2+}, and 100 μM for RR-treated mitochondria), the transient activation of State 4 respiration is observed at given experimental conditions.

Thus, based on the results presented here, we propose that modulating sites involved in the above-mentioned effects of Cd^{2+} (stimulation of State 4 respiration, inhibition of DNP-uncoupled respiration, and disappearance of the respiratory response upon ADP addition, or complete uncoupling) are located on the matrix side of the membrane as indicated presumbly by others (Sanadi *et al.*, 1981; Rasheed *et al.*, 1984; Chavez *et al.*, 1985). The most intriguing fact is that the inhibition of State 3 respiration by cadmium in the presence of RR (Fig. 3) took place even at low concentrations of cadmium chloride in the buffered medium. This is distinct from three mentioned effects of Cd^{2+},which were found in the presence of RR only at very high concentrations of Cd^{2+}. From these data, it appears likely that there is one more site affected by Cd^{2+}, which modulates oxidative phosphorylation and located on the cytoplasmic face of the inner membrane. Therefore, our evidence gives additional support to the previous suggestion of others (Yagi and Hatefi, 1984; Lippe *et al.*, 1988) concerning the possible existence of a separate dithiol site on the external side of the inner membrane that affects oxidative phosphorylation. In connection with

this, our observations that 100 μM of Ba^{2+}, Sr^{2+} (Mn^{2+}) or Ca^{2+} per se decrease State 3 respiration in the absence of Cd^{2+} in the incubation medium and the value of this inhibition corresponds to that obtained in the presence of cadmium chloride alone at its concentrations of 2.5, 5, and 7.5 μM, respectively, become more substantial. The evidence on the inhibition of State 3 respiration by divalent cations under consideration, namely by Sr^{2+} (Caplan and Carafoli, 1965), Ba^{2+} (Diwan, 1985), Ca^{2+} (Moreno-Sanchez, 1985), and Mn^{2+} (Gavin *et al.*, 1992) at similar concentrations are described in the literature as well. There are also analogous data for RR (Vashington *et al.*, 1972). Thus, in our mind, the inhibitory effect of Cd^{2+} on ADP-stimulated respiration is a direct one and is not due to the other Cd^{2+} effects described above.

The data on respiration are consistent with the evidence found by us in the swelling experiments. We have shown (Figs. 4 and 5) that starting from 10 μM, Cd^{2+} induces increased permeability of the inner membrane of nonenergized rat liver mitochondria to K^+ and H^+ in nitrate media, which is only enhanced in NH_4NO_3 medium upon energization of the mitochondria. These alterations of the ionic permeability are eliminated by RR present in the incubation medium from the start of experiment. In addition, they are activated by Ca^{2+} and inhibited by Ba^{2+}, Mn^{2+}, and Sr^{2+} (with respectively increasing degrees). The last effect (inhibition of Cd^{2+}-induced swelling), however, is observed only in NH_4NO_3 medium and only after energization (Fig. 4). Stimulation of K^+ uptake by energized rat liver mitochondria in K-acetate medium under the influence of Cd^{2+} (Fig. 6) is also enhanced by Ca^{2+}, but there is no action of the other divalent cations under test conditions [as it is the case in KNO_3 medium (Fig. 5) for K^+ permeability and in NH_4NO_3 medium in the absence of energization (Fig. 4) for H^+ permeability]. Moreover, in the presence of RR, the Cd^{2+}-induced permeabilization of the membrane only rises in the K-acetate medium in contrast to the situation found in the nitrate media. Moreover, there is no influence of all divalent cations examined (including Ca^{2+}) on the swelling produced by Cd^{2+} in the presence of RR in the K-acetate medium.

Taken together, our data imply, therefore, that there may be, at least, two separate sites (or set of sites) affected by Cd^{2+}, which are responsible for the shift of the permeability of the inner membrane to ions and are accessible to Cd^{2+}, correspondingly, from the matrix and cytosolic faces of the inner membrane. A similar view was previously proposed by Rasheed and co-workers (Rasheed *et al.*, 1984). More exciting is the fact that these sites (or set of sites) may coincide, in our opinion, with those modulating respiration changes (see above) or be vicinal to them. A role for external Ca^{2+}, i.e., with the simultaneous presence of RR in an incubation medium (Figs. 4C and 5B, traces 3), consisting likely in the increase of accessibility of vicinal protein thiol groups to the reaction with Cd^{2+} [as it seems to take place in the case of PhAsO, a bifunctional SH reagent (Kowaltowski and Castilho, 1997)], is also implied.

The above conclusions are additionally supported by the evidence, which we obtained from isotope experiments. These include the increase of the inner membrane permeability to K^+ in acetate medium in the presence of RR (as judged by the increment of $^{137}Cs^+$ uptake in this medium) produced by low Cd^{2+} concentrations, as well as the synergetic action of Ca^{2+} and the antagonistic effect of RR on Cd^{2+}-induced efflux of $^{137}Cs^+$ from the matrix after energized rat liver mitochondria attain their steady states in the presence of valinomycin (Fig. 8).

Thus, divalent cations under test interfere with practically all Cd^{2+} effects on respiratory activity and the inner membrane permeability of the mitochondria performed here. On the one hand, this may be attributed to the proposed changes in cadmium concentration in the matrix produced by these bivalent metal ions due to their competition with Cd^{2+} on the affinity sites of the Ca^{2+} uniporter (Saris and Jarvisalo, 1977; Jarvisalo *et al.*, 1980; Chavez *et al.*, 1985). On the other hand, the possible competition of these cations with other binding sites affected by cadmium, which influence respiratory function and the ionic permeability of the mitochondria (see above) is also possible. Moreover, the mode of action of these divalent cations on the Cd^{2+}-induced alterations of the mitochondrial function found here is quite similar to the modulating effects of these cations on the induction of the mitochondrial permeability transition (PT) (Zoratti and Szabo, 1995). For this reason, it may be important to recollect what is known about this issue in the literature.

Indeed, it has been realized for years that Ca^{2+} is an inducer of the PT, while Sr^{2+}, Mn^{2+}, and Ba^{2+} act as its competitive antagonists (Hunter and Ford, 1955; Hunter *et al.*, 1976; Haworth and Hunter, 1979; Hunter and Haworth, 1979; Bernardi *et al.*, 1992; Szabo *et al.*, 1992) and exert their inhibitory effects at a matrix-side site (Bernardi *et al.*, 1993). The site of action of these cations is proposed to be the same as that responsible for the activating effect of Ca^{2+}. It is interesting that the K_i values determined by Bernardi *et al.* (1992) for divalent cation inhibition of mitochondrial permeabilization (for PhAsO as inducer) in isolated rat liver mitochondria were equal to 20, 21, and 200 μM for Mn^{2+}, Sr^{2+}, and Ba^{2+}, respectively. This may be the reason that, in some cases, Ba^{2+} does not exert an inhibitory action on Cd^{2+}-produced membrane alterations found in our experiments (that the concentrations of divalent cations used here did

not exceed 100 μM, perhaps, may not a good choice in the case of Ba^{2+} to obtain a maximal effect). In turn, we have checked this possibility and, indeed, the rise of Ba^{2+} concentrations up to 250 μM in an incubation medium enhances the antagonistic ability of this metal ion (data not presented). The other possibility for the failure of Ba^{2+} to eliminate the Cd^{2+} effects on the respiration of rat liver mitochondria may be because Ba^{2+} has the largest ionic radius among the divalent cations used here. Bernardi and colleagues (Bernardi *et al.*, 1993) have also identified the inhibitory external (on the cytoplasmic face of the inner membrane) site for bivalent metal ions (including Ca^{2+}). As soon as cations are transported on the Ca^{2+} uniporter, both the inner and/or outer sites may play a role, depending on experimental conditions. Moreover, these two Me^{2+} binding sites can be experimentally distinguished (Szabo *et al.*, 1992; Bernardi *et al.*, 1993).

Therefore, in keeping in mind our findings that divalent cations influence on Cd^{2+}-induced effects on isolated rat liver mitochondria, as well as the similar ionic radii for Cd^{2+} and Ca^{2+}, we hypothesized that Cd^{2+} can interfere in the Ca^{2+}-dependent processes (studied here) by replacing Ca^{2+} in its binding sites. Of course, other ways of disturbing Ca^{2+} regulation and Ca^{2+} homeostasis by Cd^{2+} are not excluded. Finally, the indirect evidence that $\Delta\psi_{mito}$ declines in the presence of cadmium (Fig. 8B and C) (literature data describing a Cd^{2+}-induced drop of mitochondrial membrane potential *in vitro* and *in situ*; Chavez *et al.*, 1985; Martel *et al.*, 1990; Koizumi *et al.*, 1994), as well as CSA sensitivity of Cd^{2+}-stimulated swelling in all media tested here, indicate the involvement of PT pore in the processes induced by Cd^{2+} in isolated rat liver mitochondria.

In summary, the Cd^{2+}-stimulated increase of membrane ion permeability, the activation of State 4 respiration, the disappearance of the respiratory response of rat liver mitochondria to ADP addition (or complete uncoupling) produced by Cd^{2+}, the action of divalent cations tested on all these processes, the Cd^{2+}-induced $\Delta\psi_{mito}$ drop, and the susceptibility of the Cd^{2+} effects to CSA, may be explained by the possible stimulation of the opening of the PT pore by Cd^{2+}.

The elucidation of this issue and detailed study of the mechanism of damage by Cd^{2+} of mitochondrial function are under way (Belyaeva *et al.*, in preparation).

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