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EFFECTS OF Pb^{2+} ADDED *IN VITRO* ON Ca^{2+} MOVEMENTS IN ISOLATED MITOCHONDRIA AND SLICES OF RAT KIDNEY CORTEX

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Abstract—We have studied the effects of Pb^{2+} added *in vitro* on the movements of Ca^{2+} in renal cortical mitochondria and tissue slices. The isolated mitochondria rapidly accumulated $^{45}Ca^{2+}$ at 25° by a mechanism that was dependent on respiration and inhibited 96% by ruthenium red. A concentration of 10 μM Pb^{2+} inhibited the Ca^{2+} accumulation at least as effectively as did ruthenium red. About 20% of the Ca^{2+} accumulation persisted at 1° with a similar sensitivity to inhibitors, including 60% inhibition by Pb^{2+}. Similar results were obtained when the accumulation of Ca^{2+} at 25° was measured by means of a calcium-sensitive electrode, Pb^{2+} inhibiting by 80%. Calcium that had been accumulated by mitochondria at 25° was released completely by the ionophore A23187 or by 10 μM Pb^{2+}. The release induced by Pb^{2+} was greatly inhibited by ruthenium red. The Ca^{2+} content of tissue slices of renal cortex increased 4-fold during incubation at 1° while the Ca^{2+} content of mitochondria within the slices more than doubled, the latter being determined by isolation of mitochondria from the slices after incubation. The presence of Pb^{2+} (200 μM) in the incubation medium of the slices substantially reduced the entry of Ca^{2+} into the whole slices and into mitochondria within the slices. When the slices preincubated at 1° were warmed to 25° in oxygenated medium, they brought about a net extrusion of Ca^{2+}, some of which was derived from the mitochondria; Pb^{2+} did not alter the final level of Ca^{2+} then attained in the slices, but it caused a significant decrease in the quantity retained in the mitochondria. We conclude that Pb^{2+} both inhibits the uptake of Ca^{2+} by renal cortical mitochondria and displaces Ca^{2+} from them, these effects occurring whether the mitochondria are isolated or *in situ*.

Inorganic lead added to biological systems *in vitro* is known to inhibit Ca^{2+} transport activities in whole cells of brain [1] and liver [2] and in mitochondria isolated from heart [3, 4] and brain [3, 5, 6]. The energy-dependent accumulation of Ca^{2+} by isolated mitochondria is inhibited markedly by Pb^{2+} added *in vitro*, and Parr and Harris [4] found it to be the activity of heart mitochondria that was most sensitive to the heavy metal. Nevertheless, Pounds *et al.* [2], studying the distribution of Ca^{2+} in isolated hepatocytes, have shown recently that Pb^{2+} did not reduce the quantity of Ca^{2+} in a kinetically determined compartment which they believed to include the mitochondrial Ca^{2+}.

No studies have yet been made of the effects of Pb^{2+} on Ca^{2+} transport in renal preparations despite the fact that the kidney is the soft tissue which shows the greatest accumulation of Pb^{2+} during intoxication *in vivo* [7] and is a major site of plumbotoxic consequences [8–11]. We show below that Pb^{2+} added *in vitro* markedly inhibited the accumulation of Ca^{2+} by mitochondria isolated from rat kidney cortex and that it also reduced the Ca^{2+} content of mitochondria within incubated slices of renal cortex. A preliminary report of some of these results has been presented [12].

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METHODS

Male albino rats (200–250 g) of the Zivic–Miller strain of Sprague–Dawley were used. They were fed *ad lib.* on a stock diet of rat chow (Ralston Purina Co.) and water. The animals were killed by decapitation, their kidneys were removed, and the cortices were freed from medullary material while on a Petri dish cooled with ice.

Experiments with mitochondria. Mitochondria were isolated from kidney cortex by the method of Johnson and Lardy [13]. Medium for mitochondrial extraction contained 250 mM mannitol, 75 mM sucrose, 2.5 mM tris(hydroxymethyl)aminomethane (Tris), at pH 7.4, and 0.1 mM ethylenediamine tetraacetate (EDTA); this is referred to as mannitol-sucrose or MS medium. After isolation, the mitochondria were washed three times with EDTA-free MS medium, in order to remove as much EDTA as possible. Mitochondria were finally resuspended in the incubation medium, which contained 300 mM mannitol, 5 mM KCl, 2.5 mM Tris and 0.2% bovine serum albumin (Cohn fraction V, "essentially fatty acid-free", from the Sigma Chemical Co., St. Louis, MO). The medium was maintained at pH 7.4. The final volume of each preparation was usually 2.5–3.0 ml (13–15 mg protein/ml). The respiratory activity was routinely checked with an O_2 electrode, and the preparation was used for further work only if the respiratory control ratio with pyruvate *plus* malate as substrate was at least 5.0.

The study of the mitochondrial uptake of Ca^{2+} was carried out by determining the accumulation of $^{45}Ca^{2+}$ or by using a calcium-selective electrode. The uptake of $^{45}Ca^{2+}$ was usually studied at 25° although a few experiments were carried out at 1°. The mitochondria were preincubated for 5 min in the mitochondrial incubation medium. The uptake was initiated by addition of $^{45}Ca^{2+}$ (0.3 μCi) to give a total Ca^{2+} concentration of 50 μM and was stopped after an appropriate time interval (between 5 sec and 30 min) by pipetting 0.1 ml of incubation suspension onto a Millipore filter (0.45 μm pore diameter) seated on a vacuum filtration manifold at reduced pressure (−120 mm Hg). The mitochondria retained on the filter papers were then washed immediately with 15 ml of ice-cold washing medium (2.5 mM Tris, 300 mM mannitol, 5 mM KCl, 0.2% albumin, and 100 mM KCN) containing unlabeled Ca^{2+} (50 μM). This procedure took 2–3 sec. Radioactivity retained by the washed filters was measured in a liquid scintillation spectrometer (Packard Tri-Carb Scintillation Spectrometer, model B-2450), using the external standard ratio to correct for quenching.

In other experiments, a calcium-sensitive electrode (Instech Laboratories, Glenn Mills, PA) was used in conjunction with a calomel electrode as a reference. The electrode was coupled to a high impedance input amplifier (Instech Laboratories) and then to a recorder (Kip & Zonen, model BD40). The electrodes were dipped into 5 ml of reaction medium which was contained in a water jacketed chamber (25°) and stirred with a magnetic bar. To test the specificity of the electrode for Ca^{2+}, its response to a number of other cations present in the experiments was tested. The sensitivity of the electrode for Ca^{2+} was at least fifteen times greater than for Pb^{2+}, Na^+, Mg^{2+}, H^+ (Fig. 1) and K^+ (not shown).

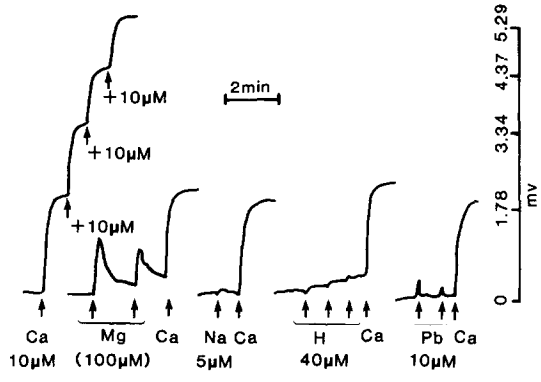

Fig. 1. Pen recordings showing the responses of the Ca^{2+}-sensitive electrode to the addition of various cations to the medium (2.5 mM Tris, 300 mM mannitol, 5 mM KCl and 0.2% albumin). The Ca^{2+}-sensitive and reference electrodes were placed in 5 ml of medium at 25°; the medium was contained in a water jacketed chamber and stirred with a magnetic bar. For calibration, 10 μl amounts of 5 mM $CaCl_2$ were added, giving 10 μM increments. Then 10 μl volumes of $MgCl_2$, $CaCl_2$, NaCl, HCl or $Pb(NO_3)_2$ were added from stock solutions so as to increase the concentrations by the amounts indicated.

Experiments with slices. The renal cortices from a single rat were cut into slices 0.2 to 0.3 mm thick [14], and the slices were then randomly divided over two beakers of Ringer medium (see below), one set to be used for treatment with Pb^{2+} and the other to serve as a control; the medium was maintained at 1°. After 15 min, the slices were transferred in groups weighing 50–100 mg to Erlenmeyer flasks (25 ml capacity) each containing 3 ml of the medium. The preincubation at 1° was then continued for a further 75 min, to make 90 min in all. During the last 15 min of this period, the flasks were gassed with O_2 before being stoppered. At the end of the preincubation period, two to three vessels were removed, and their slices were collected for analysis and, in some experiments, for isolation of their mitochondria. The remaining flasks were transferred to a shaking water bath at 25° for incubation of the slices. At intervals, two to three flasks were removed, and their slices were collected for analysis and/or isolation of mitochondria.

The Ringer medium contained: 146.5 mM Na^+, 5.0 mM K^+, 1.2 mM Ca^{2+}, 1.0 mM Mg^{2+}, 162.7 mM Cl^-, 1.0 mM SO_4^{2+}, 10.0 mM tris(hydroxymethyl)-aminomethane (pH 7.0) and 10.0 mM glucose. Lead was added as $Pb(NO_3)_2$, unless otherwise noted, and to the control media was added the same concentration of NO_3^-, as $NaNO_3$.

After incubation, the contents of each flask were tipped onto a hardened filter paper (Whatman No. 54) supported on a sintered glass funnel under suction. The slices to be analyzed were then blotted and transferred to tared weighing bottles. Dry weight was determined by drying the tissue at 110° for a minimum of 4 hr [15]. Ca^{2+} contents were determined in extracts made by digesting dried tissue with 5 ml of 0.1 N HNO_3, for a minimum of 4 hr at room temperature [16]. The assay was performed by standard techniques of atomic absorption spectrometry (Perkin–Elmer, model 303).

For isolation of mitochondria, the slices from the filter paper were gently scraped directly into a homogenization vessel containing isolation medium modified by the presence of 0.2 mM La^{3+}; this prevents redistribution of Ca^{2+} into the mitochondria upon distribution of the cells [17]. The isolation then followed the procedure described above.

Expression of results. Results are given as mean ± standard error of the mean (number of observations). Differences between treatments were examined for statistical significance by Student's *t*-test, a P level of 0.05 or less being taken as signficant.

RESULTS

Isolated mitochondria. Mitochondria isolated from kidney cortex rapidly accumulated Ca^{2+} when incubated at 25° in the absence of phosphate, ADP and exogenous oxidizable substrates. Figure 2 shows that the uptake, as determined from the movement of $^{45}Ca^{2+}$, was especially rapid during the first 1–2 min after the addition of 50 μM Ca^{2+} (0.3 μCi $^{45}Ca^{2+}$) to the incubation medium. A maximum content of 220 nmoles Ca^{2+}/mg mitochondrial protein was attained after 4 min and was maintained at least up to 10 min. The uptake was inhibited by 96% when

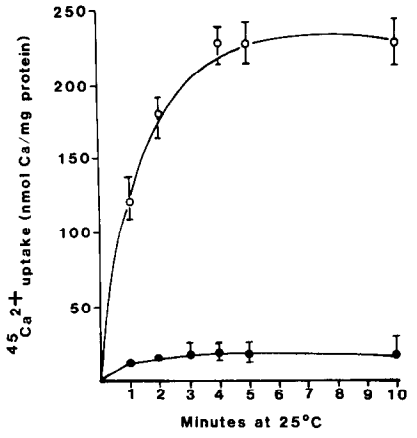

Fig. 2. Time course of $^{45}Ca^{2+}$ entry into kidney cortex mitochondria at 25°. Mitochondria were isolated from rat kidney cortex, and uptake of $^{45}CaCl_2$ was assayed at 25°. The mitochondria were preincubated for 1 min in a medium containing 10 mM Tris, 300 mM mannitol, 5 mM KCl and 0.2% albumin. The reaction was initiated by adding 50 μM (0.3 μCi) $^{45}Ca^{2+}$ and stopped by adding 15 ml of ice-cold preincubation medium containing 0.1 M KCN. The mitochondria were then separated from the medium by filtration through Millipore filters (0.45 μm pores). Radioactivity retained by the washed and dried filters was measured in a liquid scintillation spectrometer. Key: (○) control, and (●) ruthenium red (1 μM).

1 μM ruthenium red (Fig. 2) or a respiratory inhibitor such as 0.8 mM N_3^- (not illustrated) was present, indicating that the inhibitor sensitivity of Ca^{2+} accumulation by mitochondria from this tissue was similar to other tissues [18].

The effect of the presence of Pb^{2+} was studied on the same preparations of mitochondria as those of Fig. 2 and is illustrated with an expanded ordinate in Fig. 3. To facilitate a comparison of the two figures, the uptake in the presence of 1 μM ruthenium red (without Pb^{2+}) is repeated in Fig. 3. In the presence of 10 μM Pb^{2+}, there was an uptake of Ca^{2+} which, during the first 60 sec, was more rapid than that observed in the presence of ruthenium red, although much slower than in the controls of Fig. 1. At longer times, the $^{45}Ca^{2+}$ content in the presence of 10 μM Pb^{2+} declined, and the final level was only about half that seen with ruthenium red. Similar results were obtained when the Pb^{2+} concentration was 25 or 100 μM, except that the initial peak of $^{45}Ca^{2+}$ uptake became progressively less marked. The presence of 1 μM ruthenium red and 0.8 mM N_3^- together with 10 μM Pb^{2+} gave results very similar to those with 100 μM Pb^{2+} alone (Fig. 3). The marked inhibition of Ca^{2+} uptake by 10 μM Pb^{2+} is unlikely to be secondary to the inhibition of energy metabolism, because this concentration of Pb^{2+} inhibits respiration by only 20% (S. C. Kapoor and G. D. V. van Rossum, manuscript in preparation).

As a result of the experiments with cortical slices described below, it became important to examine the ability of mitochondria to accumulate Ca^{2+} at 0–1°. Chance [19] stated that liver mitochondria are able to do so, but no other results seem to be available. Our experiments were conducted as described above, except that the incubation was carried out at 1° for a single, standard time of 5 min after the addition of 50 μM Ca^{2+}. A marked uptake of Ca^{2+} was observed under control conditions, amounting to about 20% of that seen after the same incubation time at 25°. This uptake was greatly inhibited by ruthenium red, La^{3+} or N_3^- (Table 1), indicating that it had a similar energy dependence and inhibitor sensitivity to that seen at higher temperatures. A low concentration of Pb^{2+} (10 μM) also greatly inhibited the uptake, although significantly less than ruthenium red or La^{3+} (Table 1).

We carried out further experiments on the Ca^{2+} uptake at 25° by recording the changing levels of

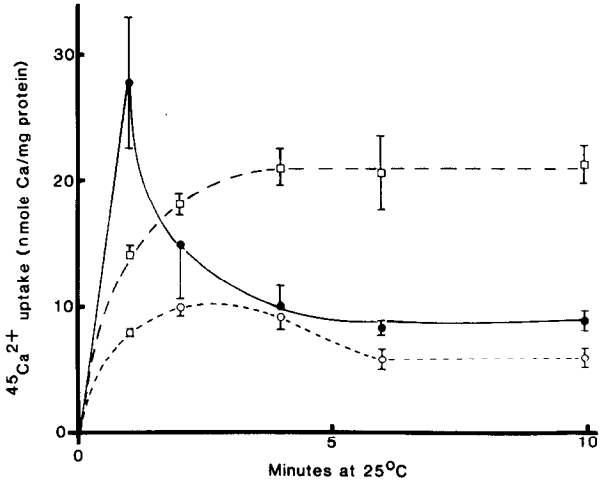

Fig. 3. Effect of Pb^{2+} on the uptake of $^{45}Ca^{2+}$ by renal cortical mitochondria at 25°. The experiments were carried out with the same mitochondrial preparations as those of Fig. 2, and the results with ruthenium red alone are the same as those illustrated there. When added, Pb^{2+} was present throughout the 1-min period of preincubation and the experimental period. Key: (●) 10 μM Pb^{2+}, (○) 100 μM Pb^{2+}, and (□) 1 μM ruthenium red.

Table 1. Ca^{2+} uptake by kidney cortex mitochondria at 1°*

Additions to medium	Total Ca^{2+} (nmoles/mg protein)	Carrier-dependent uptake of Ca^{2+}† (nmoles/mg protein)
None	19.8 ± 2.1	17.1 ± 1.4
Ruthenium red, 3 μM	2.7 ± 1.2	0
$LaCl_3$, 200 μM	2.7 ± 1.0	0.0 ± 0.04
NaCl, 500 μM	18.0 ± 1-6	15.3 ± 1.5
$Pb(NO_3)_2$, 10 μM	7.5 ± 1.7	4.8 ± 1.3
NaN_3, 15 mM	4.8 ± 1.3	2.1 ± 0.6

* Uptake was assayed with $^{45}Ca^{2+}$. The mitochondria were preincubated for 5 min in the presence of inhibitors as indicated. The reaction was initiated by adding 50 μM (0.3 μCi/ml) $^{45}CaCl_2$ and was stopped 5 min later by filtration and washing. Further details are given in Methods.

† Total Ca^{2+} accumulated in the absence of ruthenium red *minus* accumulation in the presence of ruthenium red.

Ca^{2+} in the incubation medium, with a calcium ion-sensitive electrode. These experiments differed from those described above in that the reaction mixture contained 10 mM glutamate *plus* 1 mM L-malate as respiratory substrates, Ca^{2+} was used at 100 μM, and the reaction was initiated by addition of mitochondria. In a control experiment, the mitochondria accumulated Ca^{2+} to the extent of 85 nmoles Ca^{2+}/mg protein in 5 min, with saturation clearly not yet attained (Figs. 4A and 5D). The addition of the Ca^{2+} specific ionophore, A23187 [20], caused a rapid release of all the previously accumulated Ca^{2+} in less than 2 min (Fig. 4B). The addition of N_3^- instead of A23187 (Fig. 4C) inhibited the further uptake of Ca^{2+}, but no release of Ca^{2+} was seen during the first 6 min after addition; at longer times a very slow release occurred (this part of the curve is not shown). The initial uptake of Ca^{2+} from the medium was abolished if the medium contained 5 μM ruthenium red (Fig. 4D). In the presence of 10 μM Pb^{2+} (Fig. 5A), the maxiumum uptake of Ca^{2+} by the mitochondria was reduced to 15.8 nmoles Ca^{2+}/mg protein after 5 min (18.5% of control, Fig. 5D). The delayed addition of Pb^{2+} or 5 μM ruthenium red had no significant effect on the small amount of Ca^{2+} that had been taken up in the presence of the other agent (Fig. 5, B and C).

The above experiments were all conducted under conditions in which mitochondria with only endogenous Ca^{2+} were induced to accumulate extra Ca^{2+} from the medium so that the effects of Pb^{2+} must necessarily have been due largely to an inhibition of some aspect of the accumulation mechanism. In further experiments, we attempted to see whether Pb^{2+} could also induce a displacement of mitochondrial Ca^{2+} from internal sites. Mitochondria were permitted to accumulate Ca^{2+} from the medium for 5 min, attaining a content of 50 nmoles Ca^{2+}/mg pro-

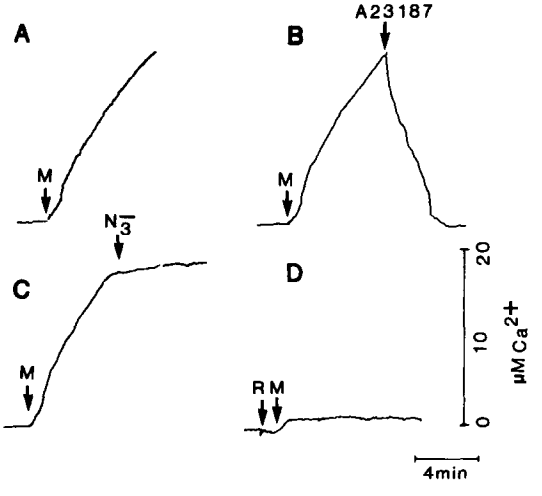

Fig. 4. Effect of Pb^{2+} and other agents on Ca^{2+} uptake by isolated mitochondria as measured by Ca^{2+}-selective electrode. The upward movement of traces indicates an entry of Ca^{2+} into the mitochondria. The mitochondria were incubated in a medium containing 10 mM glutamate plus 1 mM malate, 300 mM mannitol, 10 mM Tris, 5 mM KCl and 0.2% albumin, in the presence of 100 μM $CaCl_2$. Additions were made as indicated by the arrows: M, mitochondria: A23187, 2 μM; N_3^-, 0.8 mM: and R, ruthenium red, 5 μM.

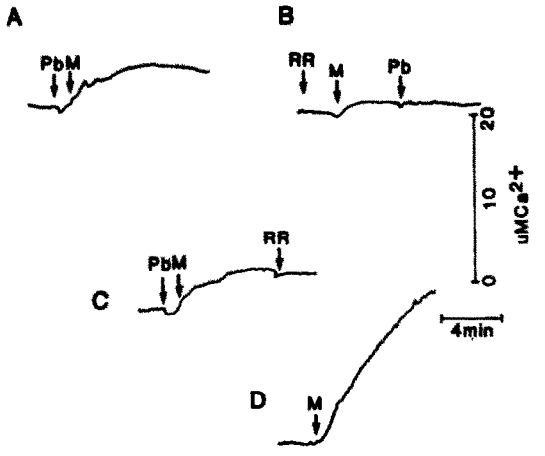

Fig. 5. Measurements of Ca^{2+} uptake in the presence of Pb^{2+} and ruthenium red with Ca^{2+}-selective electrode. The upward movement of traces indicates an entry of Ca^{2+} into the mitochondria. Conditions were as in Fig. 4. Additions at the arrows were: M, mitochondria: Pb, 10 μM; and RR, ruthenium red, 5 μM.

tein. Addition of 10 μM Pb^{2+} at this point prevented further uptake and induced the release of accumulated Ca^{2+} after a lag period of less than 1 min (Fig. 6A). All the accumulated Ca^{2+} was released into the medium in less than 4 min. The initial rapid decline of the trace on introduction of Pb^{2+} was an addition artifact and the subsequent lag period was probably due to the time required for Pb^{2+} to enter the mitochondria in sufficient quantity to displace Ca^{2+}. The addition of ruthenium red after 5 min also produced total inhibition of uptake and a later release of Ca^{2+} but the rate of release was much slower than that induced by Pb^{2+} (Fig. 6B). A similar effect of ruthenium red has been reported with liver mitochondria [21]. The addition of 10 μM Pb^{2+}, 1 min after ruthenium red, did not accelerate the slow release of Ca^{2+} (Fig. 6C). Thus, a ruthenium red-sensitive transport system is somehow involved in the lead-induced release of Ca^{2+}.

Slices of renal cortex. Two aspects of Ca^{2+} movements, and the effects of Pb^{2+} upon them, were studied in the tissue-slice preparation, namely the changes occurring in the cellular Ca^{2+} content when metabolic activity was reduced by incubation at a low temperature (1°) and the subsequent extrusion of Ca^{2+} initiated by reincubating the slices at 25°.

Incubation at 1° leads to a loss of K^+ and a gain of H_2O, Na^+, Cl^- [22, 23] and Ca^{2+}, with little change of Mg^{2+} [24]. In the present experiments there was a nearly 4-fold increase of slice Ca^{2+}, from the fresh tissue level of 8.3 to an incubated level of 31.2 mmoles Ca^{2+}/kg dry wt during a 90-min incubation at 1°. Table 2 shows that this increase was reduced substantially by the respiratory inhibitor, CN^-, and by the presence of 200 μM Pb^{2+} in the medium. The latter was equally effective as the acetate and nitrate. Used as a control, sodium acetate caused no significant alteration of the accumulation of Ca^{2+} at 1°.

To see whether the Ca^{2+} content of mitochondria contributed to the changes in whole slice Ca^{2+} at 1°, the mitochondria were isolated from samples of the slices after incubation, using La^{3+} in the homogenization medium. The central column of Table 2 shows that the mitochondria isolated after slices had been incubated at 1° contained a much greater

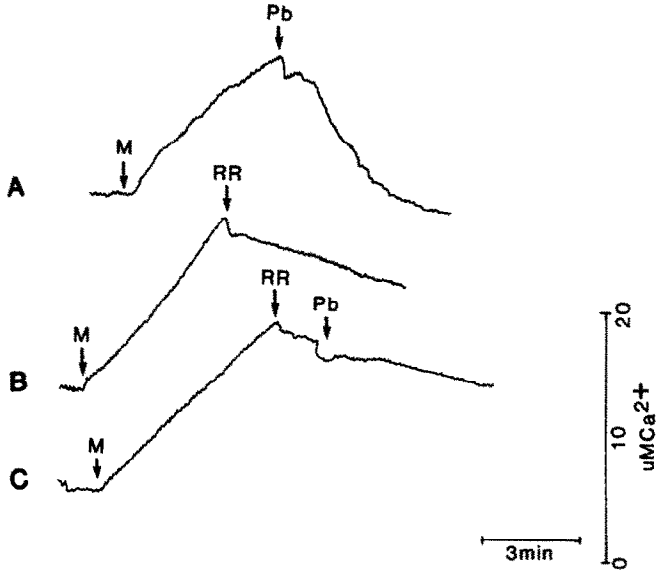

Fig. 6. Effect of Pb^{2+} and ruthenium red on the release of Ca^{2+} from the kidney cortex mitochondria, studied by Ca^{2+}-selective electrode. Upwards movements of traces indicate an entry of Ca^{2+} into mitochondria. General procedures were as in Fig. 4. Mitochondria were allowed to accumulate approximately 50 nmoles Ca^{2+}/mg protein before additions were made: Pb, 10 μM; and RR, ruthenium red, 5 μM.

Table 2. Effect of Pb^{2+} on net movement of Ca^{2+} in slices of kidney cortex and mitochondria isolated from the slices*

Additons to medium	N	Ca^{2+} contents—Slices (mmoles/kg slice dry wt)	Ca^{2+} contents—Mitochondria (mmoles/kg mitoch. protein)	Ca^{2+} contents—Mitochondria (mmoles/kg slice dry wt)
Fresh tissue	4	8.3 ± 0.3	20.1 ± 0.8	
None	12	31.2 ± 1.8	48.0 ± 1.2	12.1 ± 0.2
Sodium acetate (200 μM)	12	29.5 ± 2.3	44.3 ± 1.2	11.9 ± 0.1
Lead acetate (100 μM)	6	25.3 ± 1.9		
(200 μM)	12	21.2 ± 1.9	28.5 ± 0.7	8.3 ± 0.2
$Pb(NO_3)_2$ (200 μM)	12	20.1 ± 2.1	33.7 ± 1.4	8.5 ± 0.2
NaCN (2 mM)	12	22.3 ± 1.9	29.9 ± 1.3	7.0 ± 0.3

* Incubation of the slices was for 90 min at 1° in Tris-buffered Ringer solution, in the presence of lead salts and inhibitors. Experimental details are given in Methods. The results shown are mean ± S.E.M.

amount of Ca^{2+} than mitochondria isolated directly form fresh tissue. The accumulation of Ca^{2+} was at least partly due to an energy-dependent process, as indicated by its partial inhibition when CN^- was present in the incubation medium of the slices. The Ca^{2+} entry into the mitochondria was also reduced, by about 40%, when the slices were incubated with 200 μM Pb^{2+}. The right-hand column of Table 2 expresses the quantity of Ca^{2+} recovered in the mitochondrial preparation per unit of slice dry wt, thus allowing comparison of the mitochondrial Ca^{2+} with the total Ca^{2+} content of the slices (shown in the left-hand column). Under each set of incubation conditions, the mitochondrial Ca^{2+} accounted for about 40% of the total slice Ca^{2+}; however, this is a minimal estimate of the mitochondrial contribution, for the recovery of mitochondria from the slices was unlikely to have been complete.

When the cold-incubated slices were warmed to 25° and the medium was gassed with O_2, a net loss of Ca^{2+} resulted from reactivation of the transport system that extrudes Ca^{2+} across the plasma membrane, the net loss being largely complete after 5 min (Fig. 7). A net extrusion of Ca^{2+}, with a similar time-course, also took place when the medium contained 200 μM Pb^{2+} throughout the incubation periods at 1° and 25° but, whereas the net loss of Ca^{2+} amounted to 21 mmoles/kg dry wt in the control slices, it was only 9.5 mmoles/kg in slices incubated with Pb^{2+} (these values being compared after 60 min at 25°). However, in each case the final level of Ca^{2+} attained in the slices was similar, being 13.7 ± 0.7 (N = 12) mmoles/kg dry wt in the control slices and 14.4 ± 0.4 (N = 12) mmoles/kg in the presence of Pb^{2+}.

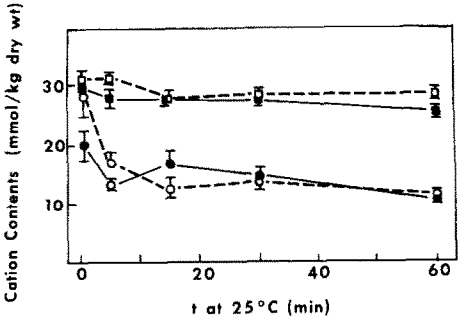

Fig. 7. Time-course of the changes of Ca^{2+} and Mg^{2+} contents in renal cortical slices in the presence and absence of 200 μM Pb^{2+}. Ca^{2+} content: (○) control, and (●) with Pb^{2+}; Mg^{2+} content: (□) control, and (■) with Pb^{2+}. Values are the mean ± S.E.M. of nine observations.

Figure 7 also shows that 200 μM Pb^{2+} had no effect on the Mg^{2+} content of the slices after either incubation at 1° or during incubation subsequently at 25°.

Mitochondria isolated from slices incubated throughout in the absence of Pb^{2+} showed a marked reduction of Ca^{2+} content during a 30-min incubation at 25° (Table 3). Thus, as in liver cells [17], the total loss of cellular Ca^{2+} upon restoration of metabolic activity was drawn partly from mitochondrial Ca^{2+}. When the calcium-loaded slices were transferred to medium at 25° containing 200 μM Pb^{2+}, the loss of mitochondrial Ca^{2+} during the 30-min incubation was increased substantially even though the total slice content of Ca^{2+} was not affected by the presence of Pb^{2+} (Table 3). This shows that Pb^{2+} was able to displace Ca^{2+} from mitochondria *in situ* in much the same way as it did from isolated mitochondria (Fig. 6).

DISCUSSION

Our experiments have shown that Pb^{2+} added *in vitro* was able both to inhibit the active accumulation of Ca^{2+} by mitochondria isolated from kidney cortex and to displace Ca^{2+} that is already present in these organelles. Furthermore, Pb^{2+} reduced the entry of Ca^{2+} into slices of the tissue incubated at 1°, at least partly as a consequence of a reduced uptake by the mitochondria *in situ*, and also displaced Ca^{2+} from mitochondria in the slices at 25°.

Lead has been shown previously to inhibit the uptake of Ca^{2+} by mitochondria from heart [3, 4] and brain [3, 5, 6]. In the heart organelles, at least, Ca^{2+} accumulation was inhibited by lower concentrations of Pb^{2+} than were required to inhibit respiration [4] and we have made similar observations with renal cortical mitochondria, for 10 μM Pb^{2+} completely inhibited the ruthenium red-sensitive Ca^{2+} uptake while causing only a small (20%) inhibition of respiration (S. C. Kapoor and G. D. V. van Rossum, manuscript in preparation). It therefore appears that the inhibition of Ca^{2+} accumulation by Pb^{2+} is not secondary to a reduction of energy metabolism but

Table 3. Displacement by Pb^{2+} of mitochondrial Ca^{2+} *in situ**

Incubation	Medium Pb^{2+} (μM)	Mitochondrial Ca^{2+} (mmoles/kg protein)	Slice Ca^{2+} (mmoles/kg dry wt)	N
90 min at 1°	0	43.5 ± 3.0†	20.9 ± 1.3	4
90 min at 1° followed by	0	20.0 ± 2.4	11.8 ± 0.7	7
30 min at 25°	200	13.3 ± 1.1‡	10.4 ± 0.4	7

* Slices of kidney cortex were loaded with Ca^{2+} at 1° in the absence of Pb^{2+} and were subsequently incubated at 25° with or without Pb^{2+}. Incubation medium contained 200 μM $Pb(NO_3)_2$ or 400 μM $NaNO_3$. In each experiment, slices of kidney cortices (from two rats) were pooled and then divided into three lots corresponding to the three treatments. After incubation, duplicate samples of slices (approximately 100 mg wet wt each) were taken for analysis, while the remaining slices of each treatment (approximately 1 g wet wt) were homogenized in the presence of 0.2 mM La^{3+} and their mitochondria isolated. Each mitochondrial preparation was assayed in triplicate or quadruplicate for Ca^{2+} and protein. values are mean ± S.E.M. (N) where N represents the number of experiments.

† Slices from the same animals incubated at 1° in the presence of 200 μM Pb^{2+} yielded mitochondria containing 26.5 ± 2.2 (N = 4) mmoles Ca^{2+}/kg protein.

‡ Significantly different from value without Pb^{2+}, P = 0.02.

is more directly on the Ca^{2+} transport mechanism itself. Evidence that Pb^{2+} interacts directly with the Ca^{2+} carrier is provided by the competitive nature of the inhibition in heart mitochondria [4] and by our finding that Pb^{2+} is itself accumulated in renal cortical mitochondria by a mechanism that is dependent on respiration and inhibited by ruthenium red [25]. The last finding is particularly important in suggesting an interaction of Pb^{2+} with the Ca^{2+} carrier, for ruthenium red is a rather specific inhibitor of the Ca^{2+} accumulating system of mitochondria [26]. It seems most probable that Pb^{2+} inhibits Ca^{2+} accumulation by competing successfully for this carrier site.

Once inside the renal mitochondria, Ca^{2+} remains in a diffusible form, for it is released rapidly by the ionophore A23187 and slowly by ruthenium red or respiratory inhibition. Since Pb^{2+} induced a much more rapid leakage of accumulated Ca^{2+} than either ruthenium red or N_3^-, it follows that the simple inhibition of Ca^{2+} uptake by the heavy metal was not the cause of the loss of Ca^{2+} which it induced. On the other hand, the entry of Pb^{2+} into the mitochondria does appear to be crucial in view of the failure of Pb^{2+} to accelerate Ca^{2+} leakage in the presence of ruthenium red. Thus, we conclude that Pb^{2+} which has entered the mitochondria either displaces Ca^{2+} from intramitochondrial binding sites or induces a rapid Ca^{2+}–Pb^{2+} exchange across the inner membrane. The finding of a rapid release of Ca^{2+} induced by A23187 tends to argue against the former of the two alternatives because the ionophore facilitates passage of the ion through membranes rather than releasing it from binding sites.

In summary, it appears that Pb^{2+} has two effects on mitochondrial Ca^{2+} metabolism. By virtue of its affinity for Ca^{2+} carrier sites, it inhibits the undirectional uptake of Ca^{2+} and, when accumulated in the mitochondria, it induces a loss of Ca^{2+} already present.

In view of the findings, with isolated mitochondria, it was anticipated that Pb^{2+} would affect the distribution of Ca^{2+} in intact cells, at least partly by way of a reduction of the quantity of Ca^{2+} in the mitochondrial compartment. Yet Pounds *et al.* [2], using cultured hepatocytes, could find no indication that Pb^{2+} altered the Ca^{2+} content of a kinetic compartment which, from studies of respiratory inhibitors [27], they believed to include mitochondrial Ca^{2+}. We have approached this problem by a different method. By incubating cortical slices at 1° the transport mechanism(s) which normally extrudes Ca^{2+} across the plasma membrane is inhibited, while the Ca^{2+} which therefore accumulates in the cytosol can still be taken up by the transporting activity of the mitochondria, which persists (see Table 1). Under these conditions, we have shown that Pb^{2+} inhibited the entry of Ca^{2+} into the whole slices and into mitochondria within them. It is possible tht Pb^{2+} inhibits passage of Ca^{2+} through the plasma membrane, so reducing its availability in the cytosol for accumulation by the mitochondria. Many examples are known of an interaction between the two ions at the plasma membrane [1, 28–30], and we cannot exclude this as a contributory factor in the effect of Pb^{2+} to limit the entry of Ca^{2+} into mitochondria *in situ* at 1°. However, the results of our experiments in which Pb^{2+} was added after Ca^{2+} accumulation by the slices clearly show that Pb^{2+} was able to act on the mitochondria *in situ* so as to displace Ca^{2+} from them, an effect analogous to that seen in isolated mitochondria. These experiments indicate that Pb^{2+} can affect mitochondrial Ca^{2+} *in situ* at 25° as well as at 1°, leading to a reduction of the pool of Ca^{2+} in these organelles. Whether this difference from the conclusions of Pounds *et al.* [2] represents a difference between renal and hepatic cells, or is due to the different techniques employed to determine mitochondrial Ca^{2+}, remains to be shown.

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