RESPIRATION-DEPENDENT STIMULATION BY INORGANIC PHOSPHATE OF Mg^{2+} RELEASE FROM RAT LIVER MITOCHONDRIA

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Received 9 October 1976
Revised version received 17 November 1976

1. Introduction

In the absence of external Mg^{2+} and oxidizable substrates Mg^{2+} has been found to leak out of liver mitochondria. The slow Mg^{2+} release is accelerated by Ca^{2+} [1] or ADP and an uncoupler [2]. Stimulation by Ca^{2+} of Mg^{2+} outflow depends on the oxidation of endogenous substrates [3] and is prevented by ADP and the cytosolic metabolic factor of Kun and his coworkers [1,2,4].

Here we provide evidence for respiration-dependent stimulation by inorganic phosphate of Mg^{2+} release from rat liver mitochondria. Our results suggest this effect might be due to a concerted action of inorganic phosphate and endogenous Ca^{2+} on the mitochondrial structure.

2. Materials and methods

Rat liver mitochondria were prepared according to Schneider [5]. Incubations were at 37°C in a medium consisting of 75 mM KCl, 100 mM sucrose, and 20 mM Tris–HCl (pH 7.5). All other additions are indicated in the figure legends. After addition of mitochondria to the medium an aliquot of the suspension was removed for Mg^{2+} determination. This time is taken as zero in all the figures. Where indicated inorganic phosphate (2.5 mM) was added to the reaction medium. All samples taken during the incubation period were rapidly spun down (1 min, 18 000 × *g*). The supernatant was diluted and Mg^{2+} measured with a model AAS 1 atomic absorption spectrophotometer (Carl Zeiss Jena).

Mitochondrial swelling was measured at 520 nm with a recording spectrophotometer. Protein was determined by a biuret method [6]. All reagents were of analytical grade. Nigericin was a generous gift of Professor Horvath (Budapest).

Abbreviations: EGTA, ethylene glycol bis(β-aminoethylether)-*N,N'*-tetraacetic; CCCP, *m*-chlorocarbonylcyanidephenylhydrazone; NEM, *N*-ethylmaleimide.

3. Results

At 37°C mitochondrial Mg^{2+} is to a large extent lost during 20–30 min incubation in a KCl–sucrose medium (fig.1). Addition of inorganic phosphate markedly increases the rate of Mg^{2+} ejection so that generally after 2–4 min between 80% and 90% of the Mg^{2+}, relative to the control, appears in the supernatant. Inhibition of endogenous respiration by rotenone significantly diminishes the outflow of Mg^{2+}. Inhibition by rotenone can be overcome by addition of succinate. In the presence of exogenous substrates, however, the rate of Mg^{2+} release is

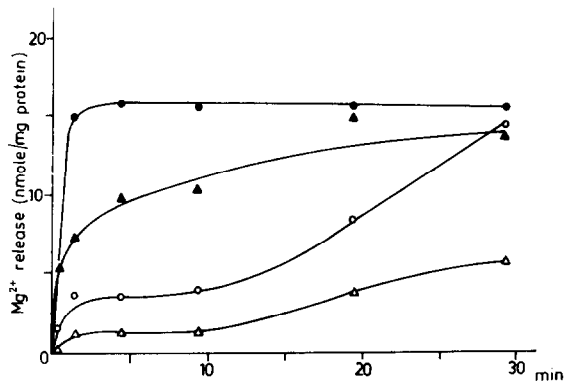

Fig.1. Effect of inorganic phosphate on the release of Mg^{2+} from liver mitochondria in the absence and presence of rotenone. 5 mg mitochondrial protein/ml. Medium see Materials and methods. Further additions to the medium: (●——●) inorganic phosphate; (△——△) inorganic phosphate and 2 μg rotenone/ml; (▲——▲) inorganic phosphate, 2 μg rotenone/ml and 10 mM succinate. (○——○) Control without any addition. For further details see Materials and methods.

markedly lower than under conditions of endogenous respiration.

The experiments summarized in fig.2 support the suggestion that phosphate uptake plays an essential role in the stimulation of Mg^{2+} ejection. NEM, a specific inhibitor of the mitochondrial phosphate carrier, decreases the rate of Mg^{2+} release as does nigericin. The latter is known for its ability to prevent phosphate uptake by dissipating the transmembranal pH-gradient, due to its action as an H^+–K^+-antiporter. In view of the experiments of Kun and his colleagues [1,3] it is of interest to see whether endogenous Ca^{2+}, which leaks out of the mitochondria during storage and incubation, affects the release of mitochondrial Mg^{2+}. As shown also in fig.2 EGTA alone decreases the rate of Mg^{2+} outflow to a level somewhat lower than that obtained with NEM. Addition of EGTA and NEM together leads to a further depression. Ruthenium red has the same inhibitory effect as EGTA.

In separate experiments (not shown here) it was found that partial removal of endogenous phosphate by preincubation with ADP decreased the rate of Mg^{2+} release as well as the total amount delivered in the absence of exogenous phosphate. Therefore, the incomplete inhibition of Mg^{2+} release by NEM or nigericin (see fig.2) might be due to the presence of endogenous inorganic phosphate.

Figure 3 shows clearly that phosphate stimulated Mg^{2+} release requires a supply of energy. Addition

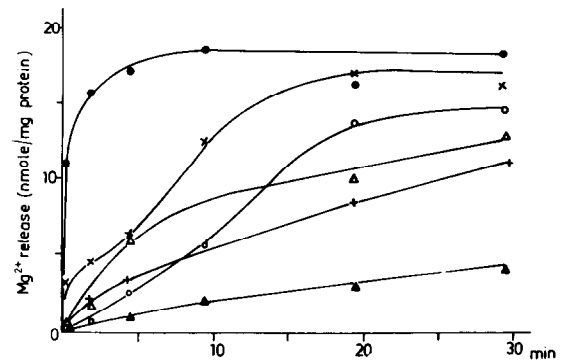

Fig.2. Inhibition by NEM and EGTA of phosphate-stimulated Mg^{2+} release from mitochondria. 5 mg mitochondrial protein/ml. Medium see Materials and methods. Further additions to the medium: (●——●) inorganic phosphate; (△——△) inorganic phosphate and 100 μM NEM; (▲——▲) inorganic phosphate, 100 μM NEM, and 1 mM EGTA; (+——+) inorganic phosphate and 1 mM EGTA; (x——x) inorganic phosphate, and 0.75 μg nigericin/mg protein. (○——○) control without phosphate. For further details see Materials and methods.

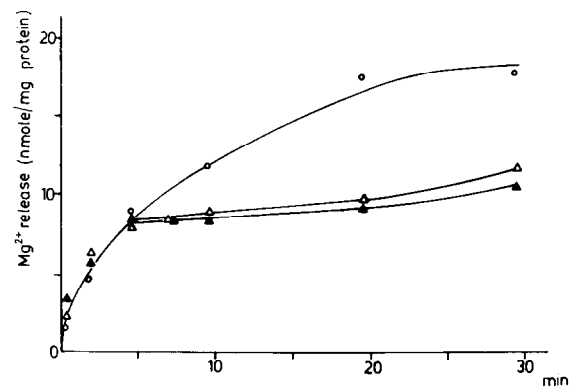

Fig.3. Effect of uncouplers and of inhibitors of electron transport on Mg^{2+} release from mitochondria. 4 mg mitochondrial protein/ml. Medium see Materials and methods. Further additions to the medium: inorganic phosphate, 2 μg rotenone/ml and 10 mM succinate. (○——○) Control. 5 min after initiating incubation 1 μM CCCP (△——△) or 10 μg antimycin A/ml (▲——▲) were added. For further details see Materials and methods.

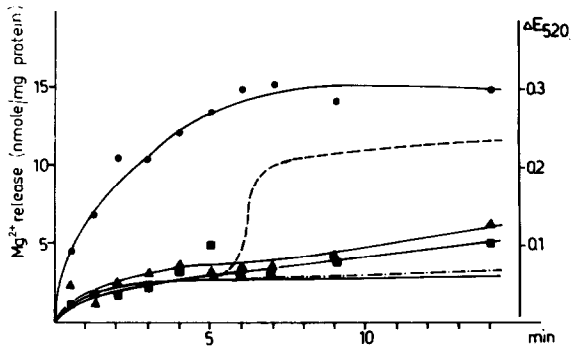

Fig.4. Stimulation by inorganic phosphate of Mg^{2+}release and swelling of liver mitochondria. Medium see Materials and methods. Further additions to the medium: inorganic phosphate (●——●, - - - -), inorganic phosphate and 2 μg rotenone/ml (▲——▲, -·-·-). Controls without any addition (■——■, ——). Mg^{2+} Efflux indicated by (●, ▲, ■); Mitochondrial swelling by (– – –, –·–·–, ——). The initial optical density in the swelling experiment was 1.66. For further details see Materials and methods.

of an uncoupler or an inhibitor of electron transport interrupts the release of Mg^{2+} that occurs in the presence of rotenone and succinate. Complete inhibition of Mg^{2+} outflow can also be achieved by adding the uncoupler or inhibitor to the medium prior to the mitochondria (data not shown).

The experiments in fig.4 were performed to rule out the possibility that the loss of structural integrity of the mitochondria is responsible for the ejection of Mg^{2+}. Only after deliverly of 70–80% of mitochondrial Mg^{2+} does a marked increase of the mitochondrial volume become evident. The same relationship between Mg^{2+} release and swelling was observed when exogenous substrates were utilized. In control experiments without inorganic phosphate or with phosphate and rotenone only a slow Mg^{2+} efflux and an insignificant swelling of the mitochondria occurred. The sequence of events excludes the possibility that swelling of mitochondria is responsible for the stimulation by phosphate of Mg^{2+} release. In accordance with the findings of Binet and Volfin [4,7] we might conclude from these data that loss of a distinct proportion of mitochondrial Mg^{2+} causes structural changes. Apparently that small portion of Mg^{2+}, which is delivered in the absence of phosphate or under conditions of inhibited respiration, comes from the intermembrane space and has no bearing on the permeability of the mitochondria. This assumption is supported by the fact that in the absence of phosphate, Ca^{2+} is retained by the mitochondria, whereas in the presence of phosphate, Ca^{2+} efflux occurs, which is delayed with respect to the delivery of Mg^{2+} (unpublished results).

4. Discussion

In a KCl–sucrose medium the release of Mg^{2+} from liver mitochondria proceeding slowly per se may be considerably stimulated by inorganic phosphate. By application of specific inhibitors of phosphate transport it could be shown that the uptake of the anion seems to be obligatory in order to achieve significant stimulation of Mg^{2+} release. Furthermore the results indicate that endogenous Ca^{2+} released during incubation supports phosphate induced stimulation of Mg^{2+} release. Thus maximum inhibition of Mg^{2+} release could only be obtained when inhibitors of phosphate and Ca^{2+} transport are added together. From the inhibitory action of EGTA and Ruthenium Red on Mg^{2+} release it may be inferred that the effect of endogenous Ca^{2+} is connected with its recycling at the mitochondrial membrane, which was demonstrated under state 4 conditions [8].

Very recently Crompton et al. [9,10] described an energy dependent Mg^{2+} release from heart mitochondria, which required the presence of inorganic phosphate. In contrast to our findings with liver mitochondria, accumulation of Ca^{2+} by rat heart mitochondria prevents the release of Mg^{2+} due to lowering of the intramitochondrial phosphate concentration.

The observation that neither with heart mitochondria [10] nor with liver mitochondria did a re-uptake of Mg^{2+} occur, is evidently due to the fact that concentrations in the medium in the mM range are necessary to obtain an influx of Mg^{2+} [10,11] whereas, when endogenous Mg^{2+} was lost, the extramitochondrial Mg^{2+} was two orders of magnitude lower than this.

It should be noticed that, in contrast to our findings with liver mitochondria, Crompton et al. [10] could not detect functional or structural alteration of rat heart mitochondria after phosphate-induced deliverly of about 60% of total mitochondrial

Mg^{2+}. With respect to beef heart mitochondria, however, the loss of more than 30% of Mg^{2+}, induced by treatment with EDTA, was always connected with a significant increase of mitochondrial volume and alteration of mitochondrial functions [12]. Schuster and Olson [13] reported a reassociation of Mg^{2+} released from beef heart submitochondrial particles. One has to take into consideration, however, that these particles are 'inside-out' so that the reuptake corresponds to loss of Mg^{2+} when compared with the sidedness of intact mitochondria. When the findings of Kun and coworkers [1,3] are compared with our results two important differences are striking. First, in our experiments inorganic phosphate is essential for stimulation of Mg^{2+} release. Second, in our experiments mobilization of Mg^{2+} depends on the energization of the mitochondria, whereas Kun and coworkers could not prevent Ca^{2+} induced Mg^{2+} release by uncouplers (see table in [1]). Concerning the supply of energy for Mg^{2+} release, our results do not so far allow us to decide whether or not the uptake of phosphate and of Ca^{2+} are the only energy requiring processes or whether extrusion of Mg^{2+} itself is energy consuming. From our results we may conclude that inorganic phosphate, in the presence of sufficient amounts of endogenous Ca^{2+}, induces a respiration-dependent delivery of mitochondrial Mg^{2+} which may be due to a concerted action of both ions at the mitochondrial membrane. Corresponding effects of Ca^{2+} and of phosphate were described with respect to the oxidation of glutamate [14] and succinate [15]. Very recently Gazzotti [16] observed that phosphate is also essential for the Ca^{2+}-induced alteration of membrane permeability for NADH.

Acknowledgements

The critical advice of Professor Carafoli (Zürich) and of Professor Hofmann (Leipzig) is gratefully acknowledged.

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