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Efflux of Magnesium and Potassium Ions from Liver Mitochondria Induced by Inorganic Phosphate and by Diamide[1,2]

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Abstract

Addition to rat liver mitochondria of 2 mM inorganic phosphate or 0.15 mM diamide, a thiol-oxidizing agent, induced an efflux of endogenous Mg^{2+} linear with time and dependent on coupled respiration. No net Ca^{2+} release occurred under these conditions, while a concomitant release of K^+ was observed. Mg^{2+} efflux mediated either by P_i or low concentrations of diamide was completely prevented by EGTA, Ruthenium red, and NEM. These reagents also inhibited the increased rate of state 4 respiration induced both by P_i and diamide. At higher concentrations (0.4 mM), diamide induced an efflux of Mg^{2+} which was associated also with a release of endogenous Ca^{2+}. Under these conditions EGTA completely prevented Mg^{2+} and K^+ effluxes, while they were only partially inhibited by Ruthenium red and NEM. It is assumed that Mg^{2+} efflux, occurring at low diamide concentrations or in the presence of phosphate, is dependent on a cyclic in-and-out movement of Ca^{2+} across the inner mitochondrial membrane, in which the passive efflux is compensated by a continuous energy linked reuptake. This explains the dependence of Mg^{2+} efflux on coupled respiration, as well as the increased rate of state 4 respiration. The dependence of Mg^{2+} efflux on phosphate transport is explained by the phosphate requirement for Ca^{2+} movement.

[1]Abbreviations: Diamide, diazenedicarboxylic acid *bis*-dimethylamide; FCCP, *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol-*bis*-(2-amino ethyl ether)-*N,N'*-tetracetic acid; P_i, inorganic phosphate; Ruthenium red, $Ru_2(OH)_2Cl_4 \cdot 7NH_3 \cdot 3H_2O$; state 4, controlled state of respiration in the presence of substrate; RCI, respiratory control index; NEM, *N*-ethyl maleimide.

[2]A partial and preliminary report of these results has been published in *Biochem. Biophys. Res. Comm.*, **78** (1977) 23.

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Introduction

Exogenous magnesium ions have long been known to protect mitochondria against swelling in salt media [1] and, in particular, against the deleterious action of calcium ions [2–6]. On the other hand, removal of endogenous Mg^{2+} by various means, ADP plus an uncoupler [7], EDTA [8], P_i [9, 10], calcium ions [11, 12], and divalent cation ionophore A23187 [13–15], brings about alterations in the membrane structure and permeability of mitochondria. Some consequences of Mg^{2+} depletion on the binding of other cations to mitochondrial membrane and on their transport have been already widely investigated [8–14]. On the contrary, the mechanism by which calcium ions [11, 12], P_i [9, 10], diamide [9], and ethacrynate [16] trigger an efflux of Mg^{2+} from mitochondria, which seems to be dependent on energy coupled respiration [9, 10], is still unclear.

The experiments reported in this paper show that low concentrations of diamide, a thiol-oxidizing agent, and 2 mM P_i induce a virtually superimposable efflux of Mg^{2+} from liver mitochondria. Such an efflux is dependent on coupled respiration, on Ca^{2+} transport and, as previously demonstrated by Crompton et al. [17] in heart mitochondria, on endogenous or exogenous P_i transport. For this reason this phenomenon will be called here "active" Mg^{2+} efflux. Evidence is also presented of a "passive" efflux of Mg^{2+} which, unlike the "active" one, is accompanied by a loss of endogenous Ca^{2+} and is apparently insensitive to Ca^{2+} and P_i transport inhibitors.

Materials and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose according to Schneider [18]. Protein concentration was determined by the biuret method [19]. Mg^{2+}, Ca^{2+}, and K^+ movements were estimated by atomic absorption spectroscopy of the supernatant [17] and cation contents at zero time were assayed in acid extracts of the pellet [20]. Oxygen uptake was measured with a Clark oxygen electrode. Uncoupler respiratory control index (URCI) was assayed under the same conditions of incubation employed for the estimation of cation movements, and with the same mitochondria preparation. At the fixed times of incubation, 1.5 μM FCCP was added, and the URCI was then calculated as the ratio of the rate of mitochondria respiration with FCCP to the rate of respiration without FCCP.

Results

Mg^{2+} Efflux Induced by Diamide

The time course of Ca^{2+} and Mg^{2+} movements across mitochondrial membrane in the presence of two different concentrations of diamide is reported in Fig. 1 and 2, respectively.

The results of Fig. 1 were obtained with 400 nmol diamide/mg mitochondrial protein, a concentration which induced an uncoupled state after about 8 minutes. Practically all calcium, initially present in the incubation medium as contaminant, was taken up rapidly both in the presence and in the absence of diamide (Fig. 1A). After about 8 minutes, when the URCI reached a value of about 2, a progressively increasing Ca^{2+} efflux from diamide-treated mitochondria was observed. No Ca^{2+} efflux from untreated mitochondria took place.

In the presence of diamide, an efflux of Mg^{2+} was constantly observed (Fig. 1B). The time course of the phenomenon was not linear, since in a second phase the rate of Mg^{2+} efflux increased concomitantly with Ca^{2+} efflux. The total amount of Mg^{2+} released from mitochondria in the external

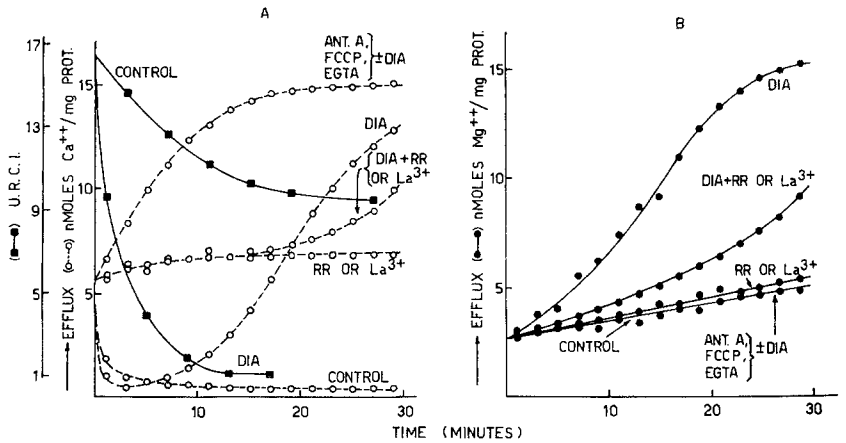

Figure 1. Effect of diamide (400 nmol/mg protein) on coupling and on the movement of endogenous Ca^{2+} and Mg^{2+}. Conditions of the control: rat liver mitochondria (1 mg protein/ml medium) was suspended in a medium containing 170 nM sucrose, 10 nM Tris-Cl pH 7.4, 5 mM Na-succinate, and 1.25 μM rotenone; temperature, 25°C. When present, 0.4 mM diamide (DIA), 5 μM Ruthenium red (RR), 10 μM $LaCl_3(La^{3+})$, 1 mM EGTA, 0.8 μM FCCP, and 1 μM Antimycin A (ANT.A). Cation contents at zero time were in nanomoles per milligram protein: calcium 18, magnesium 21.

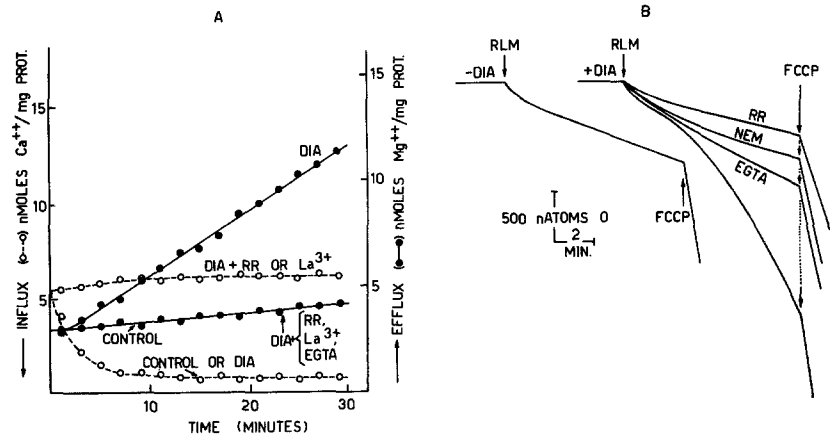

Figure 2. Effect of diamide (150 nmol/mg protein) on the movement of endogenous Ca^{2+} and Mg^{2+} (A) and on state 4 respiration (B). Experimental conditions and incubation medium as in Fig. 1. When present, 0.15 mM diamide and 50 μM NEM. Cations contents at zero time were in nanomoles per milligram protein: calcium 18, magnesium 21.

medium was approximately 70–75% of the mitochondrial Mg^{2+} (in the experiment reported in Fig. 1B, endogenous Mg^{2+} decreased from 21 to 6 nmoles/mg protein). It is noteworthy that a certain amount of Mg^{2+}, about 3 nmol/mg protein, was released spontaneously both from diamide-treated and untreated mitochondria at the moment of their suspension. However, only a minimum amount of Mg^{2+} was subsequently released from untreated mitochondria. In the presence of an uncoupler (FCCP), or a respiratory chain inhibitor (Antimycin A), diamide failed to induce any Mg^{2+} efflux. Therefore, in agreement with our previous results [9], the efflux of Mg^{2+} induced by diamide appears to be dependent on energy coupled respiration. FCCP and Antimycin A which discharge all the endogenous mitochondrial calcium (Fig. 1A) are unable per se to induce any Mg^{2+} efflux (Fig. 1B).

As shown in Fig. 1B, Mg^{2+} efflux was also prevented by EGTA (EDTA was much less efficient) and partially inhibited by Ruthenium red or La^{3+}. More precisely, Ruthenium red and La^{3+} prevented, as expected, the uptake of Ca^{2+} present as contaminant in the medium (Fig. 1A), but only partially inhibited Ca^{2+} efflux, concomitant with Mg^{2+} release, from diamide-treated mitochondria. The release of Ca^{2+}, despite the presence of Ruthenium red, can be attributed to a delayed damage of mitochondria induced by high concentrations of diamide [21, 22].

All together these results support the assumption [9], that the efflux of

mitochondrial Mg^{2+} requires previous movements of endogenous Ca^{2+}. In fact, when calcium was sequestered by EGTA outside mitochondria, or when the Ca^{2+} pump was inhibited by FCCP, Antimycin A, or Ruthenium red, Mg^{2+} efflux was also prevented.

Figure 2A shows the time course of Ca^{2+} and Mg^{2+} movements in the presence of 150 nmol diamide/mg protein, a concentration below that required to induce a complete uncoupled state. Under these conditions, even though calcium was retained within mitochondria until the anoxic state was reached, a definite efflux of Mg^{2+} occurred. However, in this case the time course of Mg^{2+} efflux was linear and not diphasic as in the presence of high diamide concentration (compare Fig. 1B and 2A). Also in these experiments, Mg^{2+} efflux was completely prevented by FCCP, Antimycin A, and EGTA (results not shown). Moreover, in the presence of low diamide concentrations, Ruthenium red and La^{3+}, which inhibit the uptake of external Ca^{2+}, completely prevented the release of both endogenous Ca^{2+} and Mg^{2+} (compare Fig. 1 and 2A).

The most remarkable difference emerging from the comparison of the results of Fig. 1 and 2A is that in the presence of high concentrations of diamide, both Mg^{2+} and Ca^{2+} are released, although not simultaneously, from liver mitochondria. In the presence of low concentrations of diamide, only Mg^{2+} was released. This indicates that Ca^{2+} and Mg^{2+} effluxes from liver mitochondria are not necessarily correlated events, and contrasts somewhat with previous results (obtained with different inducers) which suggested that Mg^{2+} accompanies the loss of Ca^{2+} [12].

Figure 2B shows that 150 μM diamide induced a significant acceleration of state 4 respiration without reaching a complete uncoupled state. Ruthenium red, EGTA, and NEM induced a strong and persistent decrease of diamide-stimulated respiration in state 4. The same reagents also inhibited the complete release of state 4 respiration induced by 400 μM diamide (results not shown).

Effect of NEM on Ca^{2+} and Mg^{2+} Efflux

Crompton et al. [17] have suggested that P_i may be required for the respiration-dependent efflux of Mg^{2+} from rat heart mitochondria. In view of a possible involvement of endogenous P_i also in the present experimental conditions the action of NEM was assayed in the presence of high and low diamide concentrations.

The results reported in Fig. 3B show that NEM completely prevented Mg^{2+} efflux from mitochondria exposed to low diamide concentrations and partially prevented Mg^{2+} efflux from mitochondria treated with high dia-

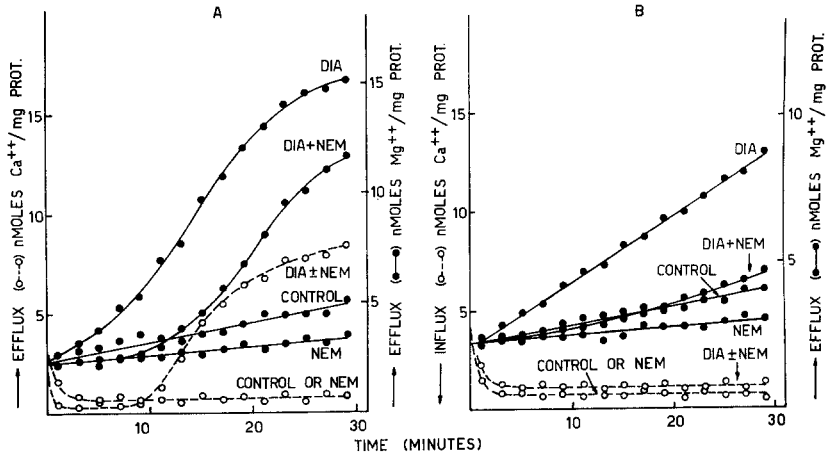

Figure 3. Action of NEM on the efflux of divalent cations induced by high (A) and low (B) concentrations of diamide. Experimental conditions as in Fig. 1. (A) 0.4 mM diamide, (B) 0.15 mM diamide. When present, 50 μM NEM. Cation contents at zero time were in nanomoles per milligram protein: calcium 12, magnesium 23.

mide concentrations (Fig. 3A). In particular, it can be observed that under the latter conditions Mg^{2+} efflux, insensitive to NEM, was concomitant with Ca^{2+} efflux, both occurring when an uncoupled state was attained. Furthermore, like Ruthenium red and EGTA, NEM inhibited the release of state 4 respiration induced by diamide (Fig. 2B). It can be concluded that only Mg^{2+} efflux dependent on coupled respiration was sensitive to NEM.

Mg^{2+} Efflux Induced by P_i

In a previous paper it was shown that in the presence of P_i both diamide and Ca^{2+} induced a respiration-dependent swelling of rat liver mitochondria, which was prevented by EGTA, Ruthenium red, and Mg^{2+} [9]. The synergic action of diamide, Ca^{2+}, and P_i is correlated with their additive effect in promoting Mg^{2+} release from liver mitochondria [9]. In the attempt to better define the movements of Mg^{2+} and Ca^{2+} induced by added P_i more detailed experiments have been carried out. The results of Fig. 4A refer to these studies and show that 2.0 mM P_i at pH 6.5 promoted Ca^{2+} and Mg^{2+} movements very similar to those induced by low diamide concentrations (compare Fig. 2A and 4A). In both cases, the efflux of Mg^{2+} was linear with time, and the initial uptake of all Ca^{2+} available in the external medium was not followed by any subsequent release. Similarly, Mg^{2+} efflux was

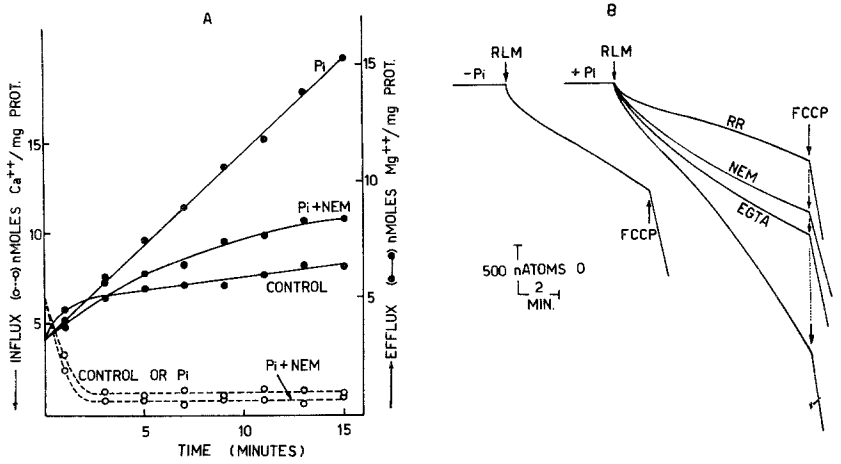

Figure 4. Effect of P_i on the movement of endogenous Ca^{2+} and Mg^{2+} (A) and on state 4 respiration (B). Experimental conditions as in Fig. 1, except for 10 mM Tris-Cl, pH 6.5. When present, 2 mM P_i and 50 μM NEM. Cation contents at zero time were in nanomoles per milligram protein: calcium 14, magnesium 24.

prevented by FCCP, Antimycin A, EGTA, and Ruthenium red (results not shown).

As expected, the P_i transport inhibitor NEM prevented Mg^{2+} efflux stimulated by added P_i. Hence, transmembrane transport of P_i seems to be a necessary condition for Mg^{2+} efflux. In the same medium, but at pH 7.5, P_i induced the same effects but with a lower rate of Mg^{2+} efflux (11.5 nmol/mg protein in 15 min). The observation that P_i induced a more rapid release of Mg^{2+} at lower pH is compatible with the possibility of an electroneutral exchange of extramitochondrial protons with mitochondrial Mg^{2+} [14].

Figure 4 B shows that EGTA, Ruthenium red, and NEM also inhibited the release of state 4 respiration induced by P_i.

Potassium Release Induced by Diamide

As shown in Fig. 5 high concentrations of diamide (400 nmol/mg protein) induced a rapid loss of mitochondrial K^+, concomitant with Ca^{2+} and NEM-insensitive Mg^{2+} release (compare Fig. 1 and 5). This K^+ efflux was almost completely inhibited by Ruthenium red (or La^{3+}) and EGTA and, unlike that of Mg^{2+}, it was not inhibited but enhanced by FCCP or Antimycin A. Figure 5 shows that K^+ efflux mediated by FCCP or

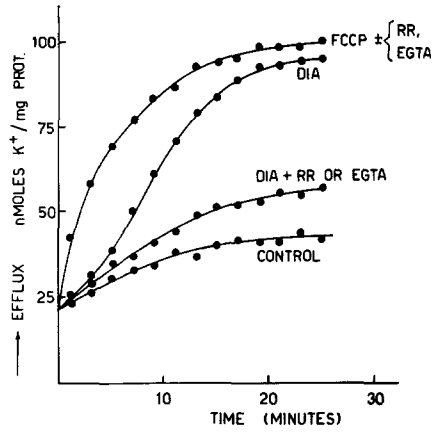

Figure 5. Effect of diamide (400 nmol/mg protein) on the movement of endogenous K^+. Experimental conditions as in Fig. 1. K^+ content at zero time: 114 nmol/mg protein.

Antimycin A, unlike that induced by diamide, was insensitive to Ruthenium red (or La^{3+}) and EGTA.

Mitochondria exposed to low diamide concentrations (150 nmol/mg protein) exhibited a less rapid release of K^+ which was not accompanied by any Ca^{2+} efflux and was completely inhibited by Ruthenium red (or La^{3+}) and EGTA (results not shown).

The sensitivity to inhibitors of Ca^{2+} transport indicates that K^+ efflux induced both by high and low concentrations of diamide is dependent, like Mg^{2+} efflux, on Ca^{2+} movements.

Discussion

The present results show that respiration-dependent Mg^{2+} efflux from liver mitochondria does not occur spontaneously as in heart mitochondria [17], but requires a proper inducer, P_i or diamide. Also low concentrations of ionophore A23187 mediate a respiration-dependent efflux of Mg^{2+} [14]. In all conditions, Mg^{2+} efflux is prevented or strongly inhibited by Ruthenium red, La^{3+}, and EGTA. This suggests that Mg^{2+} efflux is dependent on Ca^{2+} movement across mitochondrial membrane. In this respect Mg^{2+} efflux from liver mitochondria differs from that occurring in heart mitochondria, which is insensitive to Ruthenium red [17].

Ruthenium red (or La^{3+}) and EGTA both prevent Mg^{2+} efflux and strongly reduce the rate of state 4 respiration. Since the reduced rate of state 4

respiration might reflect a dampening of Ca^{2+} cycling, it is reasonable to assume that a cyclic flux of endogenous Ca^{2+} is involved in the respiration dependent Mg^{2+} efflux.

Drahota et al. [23] reported that the retention of Ca^{2+} within respiring mitochondria reflects a steady state by which the passive efflux of Ca^{2+} is compensated by an energy-linked reuptake during state 4 respiration. This energy-dissipating recycling of endogenous Ca^{2+} has been proposed by Reed and Lardy [24] to explain Mg^{2+} efflux mediated by ionophore A23187. It is possible that under the present conditions diamide, by oxidizing some pairs of thiol groups [21, 22], increases the passive efflux of accumulated Ca^{2+} and thus dissipates additional energy for the reaccumulation of this cation. An analogous mechanism can be assumed for the action of external P_i which, according to Rossi and Lehninger [25] either prevents the binding of Ca^{2+}, or causes the discharge of bound Ca^{2+}.

It is quite remarkable that chemically and functionally unrelated compounds, such as P_i, diamide, and ionophore A23187 induce the same movements of Ca^{2+} and Mg^{2+} across mitochondrial membrane. It is possible that, while acting with different mechanisms, these compounds induce significant modifications in permeability of mitochondria to divalent cations, without inducing a complete uncoupled state, which would be incompatible with the described Mg^{2+} efflux.

The involvement of P_i in Mg^{2+} efflux is confirmed by the observation that NEM inhibited not only, as expected, Mg^{2+} efflux induced by added P_i, but also that induced by diamide (Fig. 3). This would mean also that endogenous P_i plays a role, in association with Ca^{2+}, in promoting Mg^{2+} efflux. The phosphate requirement for Ca^{2+} uptake by heart and liver mitochondria, recently demonstrated by Harris and Zaba [26], is relevant to this point.

The mechanism by which accelerated recycling of endogenous Ca^{2+} promoted by P_i or diamide induces in turn an efflux of Mg^{2+}, is obscure. However, assuming that Ca^{2+} binding to inner membrane represents the first step of the mitochondrial Ca^{2+} transport [27], it is conceivable that a continuous inward and outward transport of Ca^{2+} (recycling) might interfere with Mg^{2+} binding, thus inducing a progressive displacement of this cation. This hypothesis, which is also in line with Scarpa and Azzi's findings [28] that Mg^{2+} and Ca^{2+} compete for their binding sites within mitochondrial membrane, also explains why exogenous Mg^{2+} produces a marked reduction in the rate of Ca^{2+} uptake [29].

The action of high concentrations of diamide provides a typical example of a "passive" Mg^{2+} release, even though it is preceded, in a transient phase by an "active" efflux. The "passive" release ensues when mitochondria reach

an uncoupled state and are no longer capable of reaccumulating Ca^{2+} released in the external medium. When this condition is attained, Ruthenium red becomes unable to inhibit completely Ca^{2+} efflux. This is in agreement with Sottocasa's observation that an inhibition by Ruthenium red should not be expected when the mitochondrial membrane is severely damaged [30]. Also, inhibition by NEM was incomplete under these conditions, thus indicating that, when mitochondria are damaged by high concentrations of diamide, Mg^{2+} efflux becomes independent of P_i transport.

As far as potassium is concerned, its linear time-dependent release from mitochondria treated with low diamide concentrations could be secondary, as proposed by Wehrle et al. [8], to a mobilization of K^+/H^+ antiport induced by Mg^{2+} depletion. The rapid and massive loss of potassium from mitochondria exposed to high concentrations of diamide (Fig. 5), is on the contrary an expression of true membrane damage.

From a physiological point of view, the importance of these results lies in the possibility that P_i, as well as the redox state of mitochondrial membrane thiol groups, might control Mg^{2+} and K^+ movements through modulation of the rate of Ca^{2+} recycling across mitochondrial membrane also in the intact cell.

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