Mg^{2+} Control of Respiration in Isolated Rat Liver Mitochondria†

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ABSTRACT: The role of endogenous mitochondrial Mg^{2+} as a potential regulator of mitochondrial dehydrogenase activity, and therefore of cellular respiration, was measured in isolated mitochondria containing matrix Ca^{2+} and Mg^{2+} levels resembling those occurring *in vivo.* Ca^{2+} and Mg^{2+} depletion was carried out using the cation ionophore A23187 in the presence or absence of the Ca^{2+} uniporter inhibitor ruthenium red (RR). Divalent cation depletion inhibits the oxidation of α-ketoglutarate or pyruvate in states 4 and 3, slows uncoupled respiration and results in decreased membrane potential. Since the addition of Mg^{2+} could not restore respiration, these dehydrogenases appear not to be regulated by Mg^{2+}. In contrast, similar cation depletion stimulates succinate dehydrogenase (or glutamate dehydrogenase) in state 4 without decreasing membrane potential. The addition of RR caused authentic uncoupling, accompanied by a decrease in membrane potential and an increase in membrane permeability. These effects could be completely reversed by Mg^{2+}. These and other data, showing that Mg^{2+} depletion results in a change of respiration depending on the substrate oxidized and the metabolic state, indicate that Mg^{2+} removal may have direct and indirect effects on mitochondrial respiration. A clear direct effect is the stimulation of succinate or glutamate dehydrogenase by decreasing matrix Mg^{2+}. Hence, changes in matrix Mg^{2+} (in addition to those of Ca^{2+}) could be of great consequence, not only for the control of respiration but also for metabolic pathways affected by changes in concentrations of matrix substrates.

Mg^{2+} is the most abundant divalent cation within the cell. Free Mg^{2+} in the cytosol and within intracellular organelles is only a small fraction of total Mg^{2+}, the majority being bound to proteins and cellular metabolites (Veloso et al., 1973; Corkey et al., 1986). Within the cell, mitochondria contain large amount of Mg^{2+}, some of which can be rapidly mobilized following physiological stimulation (Brierley et al., 1987; Romani et al., 1991, 1993). Little is known as to whether changes in Mg^{2+} within the mitochondrial matrix can regulate the activities of dehydrogenases and thus the overall rate of respiration. Data on the regulatory significance of Mg^{2+} for the mitochondrial dehydrogenases are scarce (Hayakawa et al., 1966; Walsh et al., 1976; McCormack & Denton, 1979; Thomas et al., 1986; Bedino & Testore, 1992): most evidence has been obtained using isolated enzymes (Hayakawa et al., 1966; Walsh et al., 1976; McCormack & Denton, 1979; Thomas et al., 1986) and/or are controversial (Hayakawa et al., 1966; McCormack & Denton, 1979). As result, for more than 20 years, research has focused on the effects of changes in matrix Ca^{2+}, rather than Mg^{2+}, on the control of mitochondrial respiration [reviewed in McCormack et al. (1990) and Hansford (1994)].

Because of the following recent observations, the time appears propitious for reopening the question of the possible physiological role of Mg^{2+}, in addition to Ca^{2+}, in controlling activities of the mitochondrial dehydrogenases: (1) the role of Ca^{2+} as the major *in vivo* regulator of the mitochondrial dehydrogenases (pyruvate dehydrogenase, α-KGDHC, and isocitrate dehydrogenase) is being questioned (Moravec & Bond, 1991, 1992); (2) mitochondrial Mg^{2+} was shown to change rapidly in response to physiological hormonal signaling (Romani et al., 1991, 1993); (3) moreover, with electron probe microanalysis it was shown that during hormonal stimulation *in vivo* Mg^{2+} but not Ca^{2+} increased in the liver mitochondria (Bond et al., 1987); (4) in contracted smooth muscle, cytoplasmic Mg^{2+} significantly decreased and mitochondrial Mg^{2+} increased, whereas mitochondrial Ca^{2+} did not significantly change during a maintained contraction (Bond et al., 1984); (5) mitochondrial free [Mg^{2+}] was shown to change reversibly during the transition of respiration from state 4 to state 3 (Brierley et al., 1987); (6) the mitochondrial spermine/Mg^{2+} ratio has been postulated to control mitochondrial respiration (Moreno-Sanchez et al., 1995); and (7) the activity of isolated α-KGDHC has been shown to be regulated not only by Ca^{2+} but also by Mg^{2+} (Panov & Scarpa, 1996).

The concentration of free Mg^{2+} within the mitochondria is in equilibrium with a larger amount of bound Mg^{2+}, thus changes in total Mg^{2+} result in changes in the concentration of free Mg^{2+} (Corkey et al., 1986). Using isolated mitochondria, a large decrease in total mitochondrial Mg^{2+} can be routinely achieved in the presence of the divalent cation

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; α-KGDHC, (α-ketoglutarate dehydrogenase complex); MOPS, 3-[*N*-morpholino]propanesulfonic acid; NAD^+, β-nicotinamide adenine dinucleotide, oxidized form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate, oxidized form; NADH, β-nicotinamide adenine dinucleotide, reduced form; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; PDHC, pyruvate dehydrogenase complex; RR, ruthenium red. Mitochondria: N-RLM, "normal" rat liver mitochondria isolated in the presence of divalent cation chelators EDTA and EGTA; S-RLM, rat liver mitochondria isolated in the sucrose medium without divalent cation chelators. Respiratory states: state 4, RLM incubated in the presence of substrates only; state 3, oxidative phosphorylation activated by addition of 0.15 mM ADP; state 3U, respiration uncoupled by addition of 0.4 μM FCCP.

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ionophore A23187 (Reed & Lardy, 1972). Reed and Lardy (1972) have shown that this ionophore released both mitochondrial Ca^{2+} and Mg^{2+}, stimulated state 4 respiration, and inhibited oxidative phosphorylation. They attributed the stimulation of respiration induced by A23187 to futile Ca^{2+} cycling. This conclusion was based on the evidence that the stimulation of the state 4 respiration induced by the ionophore was prevented by either EGTA or by inhibitors of the calcium uniporter, such as RR or La^{3+}. The conclusion was that Ca^{2+}, rather than Mg^{2+}, plays the primary role in the stimulation of the state 4 respiration induced by A23187 (Reed & Lardy, 1972) and that the role of Mg^{2+} loss was confined to inhibition of mitochondrial respiration during phosphorylation (state 3). Although this conclusion is fully supported by the data presented, the experiments have a major drawback: because the mitochondria used were isolated in the absence of Ca^{2+} chelators, their Ca^{2+} content was unphysiologically very high (Reed & Lardy, 1972).

Since the publication of the paper by Reed and Lardy (1972), a large body of evidence has been published indicating that in normal cells the mitochondrial concentrations of free and total Ca^{2+} are very low (about 0.3 μM based upon a total Ca^{2+} content of 1.1 nmol/mg of rat liver mitochondria) (Somlyo et al., 1985). The purpose of this study was to investigate the effects of the divalent cation ionophore A23187 and Mg^{2+} ions on mitochondrial respiratory activities using mitochondria having a Ca^{2+} content comparable to that present physiologically. Under these conditions, it is evident that variations in Mg^{2+} content of mitochondria affect respiration in several ways, depending upon the substrate type and the metabolic state of the mitochondria.

MATERIALS AND METHODS

Isolation of Rat Liver Mitochondria. Rat liver mitochondria were isolated by differential centrifugation from the livers of fed male Sprague-Dawley rats weighing 300−400 g (Weinbach, 1961). The medium for homogenization contained 225 mM mannitol, 75 mM sucrose, 10 mM MOPS, pH 7.2, 5 mM EDTA, 1 mM EGTA (isolation medium 1). After the first sedimentation of mitochondria, subsequent steps of the isolation procedure were performed using the medium described above, but without EDTA (isolation medium 2). These mitochondria were designated as "normal" rat liver mitochondria, or N-RLM. In some experiments, to reproduce previously published data, liver mitochondria were isolated in a medium containing 250 mM sucrose, 10 mM MOPS, pH 7.2. These mitochondria were marked as the "sucrose" rat liver mitochondria, or S-RLM. Isolated N-RLM and S-RLM were suspended, at a final concentration of 60−70 mg/mL, in isolation medium 2 and sucrose medium, respectively, and stored on ice until use. Mitochondrial protein was determined by the Biuret method (Gornall et al., 1949).

Incubation Conditions. The basic medium contained 125 mM KCl and 10 mM MOPS, pH 7.2, 25 °C. Additional details of the incubation conditions are described in the legends to the tables and figures.

Determination of Total Mitochondrial Content of Mg^{2+} and Ca^{2+}. Aliquots of a mitochondrial suspension (1 mL), containing 0.5 or 3 mg of mitochondrial protein for determination of Mg^{2+} or Ca^{2+}, respectively, were incubated for 2 min in either 125 mM KCl or 250 mM sucrose medium containing 10 mM MOPS, pH 7.2, 10 mM succinate. 1 mL aliquots were deproteinized with 10% nitric acid as described previously for atomic absorption measurement (Panov & Scarpa, 1996). This procedure was carried out using a Perkin-Elmer atomic absorption spectrometer.

Measurements of Oxygen Consumption. Mitochondrial respiratory activities were determined polarographically using a Clark electrode from Yellow Spring Co. as described by Chance and Williams (1955).

Determination of Mitochondrial Pyridine Nucleotide Fluorescence. Reduction of mitochondrial $NAD(P)^+$ was determined fluorimetrically at 25 °C using instrumentation described previously (Case et al., 1974). Fixed interference filters were used to select the excitation and emission wavelengths with the bandpaths of 340 ± 10 and 480 ± 10 nm, respectively. The fluorescence of A23187 is dramatically increased in a hydrophobic environment (Case et al., 1974), thus the background fluorescence of A23187 associated with the mitochondria was determined in fully deenergized (by addition of 0.4 μM FCCP) mitochondria, when the mitochondrial NAD(P)H fluorescence was at a minimum. The steady-state levels of mitochondrial NAD(P)H fluorescence in the presence and absence of A23187 were determined in separate experiments. Data were expressed as a percent of the maximal reduction, taken as 100%, which was calculated as the difference between the maximum and minimum levels of fluorescence of the mitochondrial NAD(P)H. The maximum level of reduction of mitochondrial pyridine nucleotides was obtained by incubating the mitochondria with 20 mM glutamate, 2 mM malate, 1 μg/mg of rotenone, or 10 mM succinate without rotenone. Full oxidation of mitochondrial NAD(P)H was obtained after 4−5 min of incubation of the mitochondria in the absence of added substrates and in the presence of the uncoupler [0.5 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)].

Measurements of Membrane Potential of Liver Mitochondria. The membrane potential ($\Delta\Psi$) was measured using a tetraphenylphosphonium (TPP^+)-sensitive electrode prepared as described elsewhere (Kamo et al., 1979). The electrode was calibrated in each incubation by adding 0.5 μM aliquots of TPP^+ to reach a final concentration of 1.5 μM. Corrections for binding of TPP^+ to mitochondria were made as described by LaNoue et al. (1986).

Measurement of Mitochondrial Swelling. Swelling of the non-energized mitochondria was monitored spectrophotometrically by recording absorption at 546 ± 3 nm at 25 °C on a strip chart recorder. To determine the inner membrane permeability to K^+ ions, media with 110 mM KNO_3 or 125 mM KCl were used, while the permeability to H^+ was determined by using 110 mM NH_4NO_3 (Henderson et al., 1969). All media were buffered with 10 mM MOPS, pH 7.2. Mitochondria (0.5 mg per 3 mL) were incubated in the absence of added respiratory substrates and in the presence of 0.5 μg of rotenone.

Statistics. Data in the Tables 1 and 2 and Figure 1 are presented as means ± standard error (M ± SE). The mean values were obtained from the data of at least five to six separate isolations of the mitochondria. With each batch of mitochondria, the mean values of Ca^{2+} and Mg^{2+} content, or respiratiory rates, were calculated as the average of two to four parallel measurements or separate experiments.

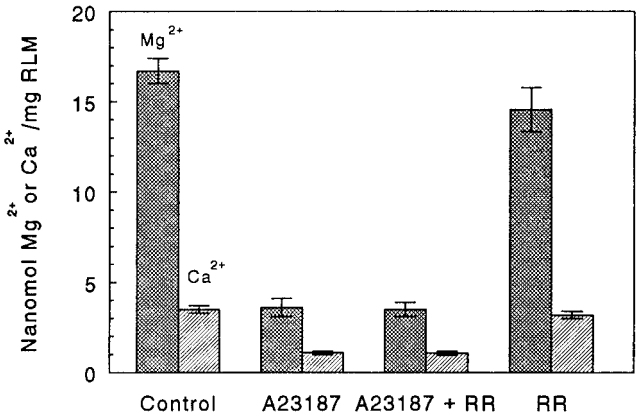

FIGURE 1: Effects of A23187 and ruthenium red on mitochondrial Mg^{2+} and Ca^{2+} contents. Incubation conditions: 125 mM KCl, 10 mM MOPS, 1 mM EGTA, pH 7.2, 10 mM succinate, 0.5 μg of rotenone/mg of N-RLM, 25 °C, for 2 min. Additions: A23187, 0.5 μM; RR, 5 μM. Left columns: mitochondrial Mg^{2+}. Right columns: mitochondrial Ca^{2+}.

Chemicals. $MgCl_2$ and $CaCl_2$ were from Aldrich Chemical Co. with 99.995% purity. The exact concentration of these cations in the stock solutions was determined by atomic absorption spectrophotometry. FCCP was obtained from the Sigma Chemical Co. (St. Louis, MO) and the stock solution of 0.04 mM was prepared using absolute 200 proof ethyl alcohol. All other chemicals were of analytical grade. Ruthenium red from Aldrich Chemical Co. was purified according to Luft (1971). All solutions were made using deionized water.

RESULTS

Mg^{2+} and Ca^{2+} Content of Rat Liver Mitochondria Isolated in the Presence or Absence of Divalent Metal Chelators. The experiments shown in Table 1 are measurements of endogenous Ca^{2+} and Mg^{2+} content of mitochondria obained in the presence or absence of chelating agents at various stages of the isolation procedure. The rationale for these experiments was to obtain mitochondria having different endogenous Ca^{2+} and/or Mg^{2+} so that rates of respiration using different substrates for matrix dehydrogenases could be compared. Table 1 shows that rat liver mitochondria isolated in 0.25 M sucrose medium (S-RLM) in the absence of added chelators have 15% higher mitochondrial Mg^{2+} content (19.2 $\pm$ 0.5 nmol/mg) than mitochondria isolated in the presence of chelators (16.7 $\pm$ 0.7 nmol/mg). The presence of 1 mM EGTA in the final incubation medium had little or no effect on the total Mg^{2+} of S-RLM and N-RLM.

By contrast, S-RLM have 4−5 times higher Ca^{2+} contents than N-RLM, depending on the presence of EGTA in the incubation medium during the Ca^{2+} determination procedure (Table 1). When incubated in the presence of 1 mM EGTA, the Ca^{2+} content of S-RLM is decreased by 4.1 nmol of Ca^{2+}. Under the same conditions no effect of EGTA is noticeable on Ca^{2+} content of N-RLM. Taken together, these measurements indicate that the presence of divalent cation chelators during the preparation of mitochondria results in relatively small changes in total Mg^{2+} content but in a several-fold decrease in Ca^{2+} content.

Table 1 also shows some of the oxygen consumption characteristics of S-RLM and N-RLM. The mitochondria isolated in the presence of chelators (N-RLM) depended upon the substrate used. With succinate as a substrate, N-RLM displayed significantly higher rates of respiration in state 4 and had similar respiratory rates to S-RLM in state 3 and state 3U (Table 1). Using other substrates, different patterns of respiration in the two preparations were observed. Hence variations in rates of respiration using different substrates would be consistent with the differences in Ca^{2+}, but not Mg^{2+}, content in these two different preparations of mitochondria.

Decreased Mitochondrial Mg^{2+} and Ca^{2+} Content in the Presence of A23187 and the Effect on Basal Respiration. To further study how depletion of Ca^{2+} and Mg^{2+} affects mitochondrial respiration, N-RLM were treated with the divalent cation ionophore A23187 in the presence of 1 mM EGTA. Since Reed and Lardy (1972) have shown that ruthenium red (RR), a noncompetitive inhibitor of the mitochondrial Ca^{2+} uniporter (Reed & Bygrave, 1974), abolished effects of A23187 on mitochondrial respiration, we also studied effects of RR on various mitochondrial functions. Figure 1 shows the effects of the ionophore and RR on Mg^{2+} and Ca^{2+} content of mitochondria. The addition of 0.5 μM A23187 to N-RLM oxidizing succinate in the presence of 1 mM EGTA results in a 78.4% and 68.6% loss of the mitochondrial Mg^{2+} and Ca^{2+} content, respectively. The extent of Mg^{2+} and Ca^{2+} release induced by the ionophore in the presence of 1 mM EGTA was similar in the presence and absence of respiratory substrates (not shown). Reed and Lardy (1972) used RR to prevent cycling of Ca^{2+} released by the ionophore. Figure 1 shows that addition of 5 μM RR after A23187 did not further affect mitochondrial Ca^{2+} and Mg^{2+} content. Interestingly, when added alone, RR caused a 12.8% decrease in Mg^{2+} content without decreasing mitochondrial Ca^{2+}.

Table 2 shows the effects of Mg^{2+} depletion on respiratory parameters of mitochondria oxidizing various substrates in different metabolic states. In the experiments of Reed and Lardy (1972), the addition of A23187 to Ca^{2+}-enriched RLM respiring in state 4 resulted in uncoupling of the mitochondria irrespectively of the substrate used (Reed & Lardy, 1972). Since this uncoupling could be inhibited by EGTA, RR, or La^{3+} in the incubation medium (Reed & Lardy, 1972), it was concluded that it was the result of a futile Ca^{2+} cycling. The data presented in Table 2 were obtained with N-RLM having a low Ca^{2+} content, comparable with that occurring physiologically. At variance with the data presented in Reed and Lardy (1972), using these mitochondria, the effect of A23187 on mitochondrial respiration was strongly dependent on the type of the substrate used.

Overall, the data support the notion that the rate of respiration of mitochondria upon depletion of Ca^{2+} and Mg^{2+} by A23187 depends on the substrate being oxidized. Table 2 (first vertical column) shows that state 4 respiration is stimulated 68% when mitochondria oxidize succinate, is unchanged when they oxidize glutamate−malate, and is inhibited when they oxidize α-ketoglutarate. The uncoupling effect could not be accounted for by futile Ca^{2+} cycling, as proposed by Reed and Lardy (1972), because (a) the extramitochondrial medium contained 1 mM EGTA; (b) this effect was not present with all substrates; and (c) further addition of RR, which effectively inhibits mitochondrial Ca^{2+} uptake, caused an additional 2−3-fold stimulation, rather than an inhibition, of respiration.

Table 1: Mg^{2+} and Ca^{2+} Content in Rat Liver Mitochondria Isolated in the Presence (N-RLM) or Absence (S-RLM) of the Divalent Metal Chelators[a]

parameters	S-RLM		N-RLM	
	no EGTA	EGTA	no EGTA	EGTA
divalent metal content (nmol/mg of RLM)				
Mg^{2+}	22.5 ± 0.7	19.2 ± 0.5	16.3 ± 0.8	16.7 ± 0.7
Ca^{2+}	18.4 ± 0.2	14.3 ± 0.4	3.8 ± 0.4	3.5 ± 0.2
respiratory rates (ng of atom O/min/mg of RLM)				
state 4	19.7 ± 2.0		26.1 ± 1.5	
state 3	93.8 ± 18		107.6 ± 6.5	
state 3U	157.8 ± 23		147.9 ± 8.3	

[a] Incubation conditions: for Ca^{2+} and Mg^{2+} measurements mitochondria were incubated for 2 min at 25 °C under constant stirring in the medium containing 125 mM KCl, 10 mM MOPS, pH 7.2, 10 mM succinate, 0.5 μg of rotenone/mg of RLM with or without 1 mM EGTA. Mitochondrial respiratory activities were measured in the same medium without EGTA, plus 2 mM P_i.

Table 2: Effects of A23187 and Ruthenium Red on Mitochondrial Respiration[a]

substrates and additions	state 4	state 3	state 3U	RCR
	ng of atom O/min/mg of RLM			
succinate plus rotenone, Mg^{2+} not present				
control	21.2 ± 2.4	145.6 ± 7.8	162.7 ± 7.0	5.4 ± 0.2
A23187	35.6 ± 3.3	50.5 ± 3.2	146.5 ± 15.0	1.4 ± 0.3
A23187 + RR	75.5 ± 4.7	88.7 ± 11	109.0 ± 7.3	1.2 ± 0.4
succinate plus rotenone, 2 mM Mg^{2+} present				
control	25.8 ± 1.8	124.8 ± 8.2	148.3 ± 5.7	4.9 ± 0.8
A23187	27.0 ± 2.7	115.2 ± 15.2	131.3 ± 6.6	4.2 ± 0.5
A23187 + RR	28.5 ± 1.0	111.9 ± 5.4	138.5 ± 4.3	3.9 ± 0.4
glutamate plus malate, Mg^{2+} not present				
control	9.7 ± 1.1	91.8 ± 4.9	91.2 ± 7.6	9.5
A23187	9.8 ± 1.6	21.8 ± 1.2	70.0 ± 3.5	2.2
A23187 + RR	38.4 ± 3.3	46.2 ± 5.4	56.2 ± 6.4	1.2
glutamate plus malate, 2 mM Mg^{2+} present				
control	10.2 ± 1.6	82.2 ± 4.3	105.8 ± 9.1	8.1
A23187	10.3 ± 2.1	60.1 ± 12.6	71.2 ± 10.6	5.8
A23187 + RR	15.6 ± 4.3	69.6 ± 11.5	90.6 ± 14.3	4.5
α-ketoglutarate + malate, 2 mM Mg^{2+} present				
control	11.0 ± 1.3	60.6 ± 3.2	74.2 ± 11.2	5.5
A23187	5.9 ± 0.9	4.9 ± 1.2	8.2 ± 3.2	0.8
A23187 + RR	4.9 ± 1.3	6.6 ± 1.5	12.4 ± 2.3	1.3

[a] Incubation conditions: 125 mM KCl, 10 mM MOPS, 2 mM KH_2PO_4, pH 7.2, 1 mM EGTA. Substrates: 10 mM succinate + 0.5 μg of rotenone; 20 mM glutamate + 2 mM malate; 10 mM α-ketoglutarate + 2 mM malate. Additions: A23187, 0.5 μM; RR, 5 μM; ADP, 150 μM; FCCP, 0.4 μM.

The addition of 5 or 50 μM RR alone did not alter rates of state 4 respiration (not shown) even if Mg^{2+} content of mitochondria decreased by 12.8% (Figure 1). However, RR further increased by 2−3-fold state 4 respiration of mitochondria oxidizing succinate or glumate−malate but not that of mitochondria oxidizing α-ketoglutarate or pyruvate (not shown).

The presence of 2 mM Mg^{2+} was effective in abolishing the increase in respiration occurring after A23187 incubation (in the presence or absence of RR) in mitochondria oxidizing succinate but had no effect in mitochondria oxidizing glutamate−malate. In addition, uncoupling was not observed when mitochondria oxidize α-ketoglutarate or pyruvate. In the presence of either substrate, the addition of A23187 caused a significant inhibition of the mitochondrial respiration in all metabolic states (Table 2).

The interpretation of the effect of Ca^{2+} and Mg^{2+} depletion on the rates of oxidation of various substrates is not straightforward because the inhibition or activation shown could be the result of multiple effects: (a) a direct effect of Ca^{2+} and/or Mg^{2+} on matrix dehydrogenases and (b) a change in substrate uptake due to changes in matrix pH or membrane potential induced by the ionophore and/or RR. These individual effects will be investigated below. Yet, the effects of Ca^{2+} and Mg^{2+} depletion on state 4 oxidation of succinate and the combined effect of A23187 and RR on the oxidation of succinate or glutamate and malate could arguably be accounted for by a depletion of mitochondrial Mg^{2+}, since the addition of 1−2 mM Mg^{2+} completely restored state 4 respiration to control rates.

Effects of A23187 and Ruthenium Red on Oxidative Phosphorylation and Uncoupled Respiration. A23187 inhibited state 3 respiration with all substrates used in this study, but the extent of inhibition had a large degree of variability, depending on the substrate used. Mg^{2+} depletion of liver mitochondria inhibited the phosphorylative oxidation of succinate by 65%, that of glutamate plus malate by 76%, and that of α-ketoglutarate, pyruvate or acetyl carnitine by 90%−95%. The addition of 2 mM Mg^{2+} to the incubation medium completely restored the rate of state 3 oxidation of succinate and increased the rates of oxidation of glutamate plus malate by 73%. By contrast, Mg^{2+} addition was ineffective in restoring state 3 oxidation rates in the presence of α-ketoglutarate (see Table 2) or pyruvate (not shown). Once again, these data, showing a restoration by Mg^{2+} of the oxidative phosphorylation in mitochondria oxidizing

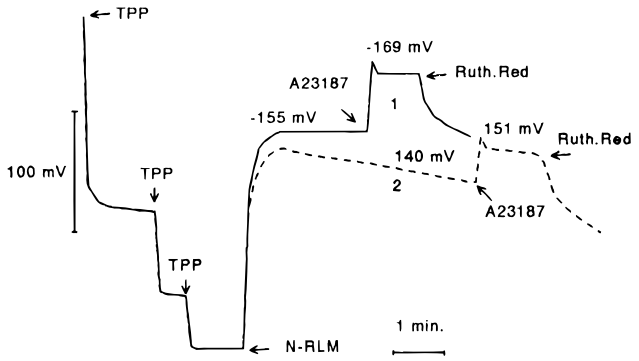

FIGURE 2: Effects of A23187 and ruthenium red on mitochondrial membrane potential. Incubation medium: 125 mM KCl, 10 mM MOPS, 1 mM EGTA, 2 mM KH_2PO_4, pH 7.2, 25 °C. Substrates: 10 mM succinate + 0.5 μg of rotenone/mg of RLM; 20 mM glutamate + 2 mM malate. Additions: TPP^+, 0.5 μM; A23187, 0.5 μM; RR, 5 μM. Trace 1: succinate. Trace 2: glutamate-plus-malate as a substrate.

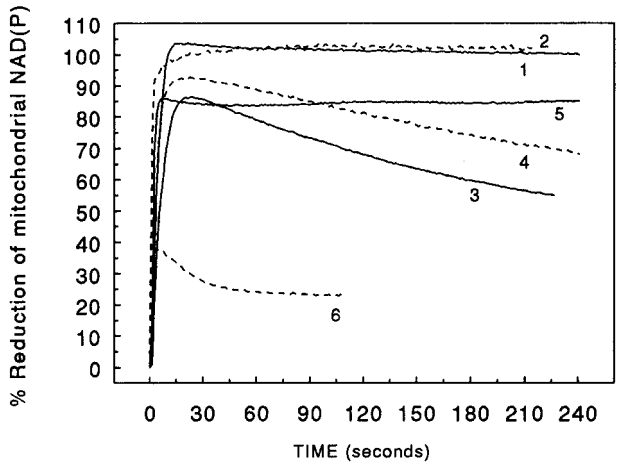

FIGURE 3: Effects of A23187 and ruthenium red on fluorescence of mitochondrial pyridine nucleotides. Incubation medium as in Figure 2. Substrates: 10 mM succinate, 0.5 μg of rotenone /mg of RLM (traces 1 and 2); 20 mM glutamate, 2 mM malate (traces 3 and 4); 1 mM pyruvate, 2 mM malate (traces 5 and 6). Traces 2, 4, and 6 were obtained in the presence of 0.5 μM A23187.

succinate or glutamate plus malate, differ from the results published by Reed and Lardy (1972), who concluded that A23187 in the absence of added Mg^{2+} completely inhibited oxidative phosphorylation with all substrates.

A23187 inhibited, although to differing extents, the oxidation of all substrates tested in mitochondria which were uncoupled by the addition of 0.4 μM FCCP. With succinate or glutamate as substrates, inhibition was more evident when both A23187 and RR were present and was largely prevented in the presence of 2 mM Mg^{2+}. However, Mg^{2+} was unable to prevent, or reverse, the A23187- or the A23187-plus-RR-induced inhibition of α-ketoglutarate (see Table 2) or pyruvate oxidation in the uncoupled mitochondria (not shown).

Effects of A23187 on Mitochondrial Membrane Potential. The data presented above demonstrate that mitochondrial depletion of Ca^{2+} and Mg^{2+} has multiple effects on respiration. To test whether stimulation of the state 4 oxidation of succinate and glutamate upon addition of A23187 or A23187-plus-RR was caused by dissipation of the membrane potential, we studied the effects of A23187 and RR on the mitochondrial electrical membrane potential ($\Delta\Psi$).

The ionophore A23187 is known to form an unprotonated complex with either Ca^{2+} or Mg^{2+}, with little interference from other divalent and monovalent cations. Because of the lipophilic properties of the complex, A23187 can catalyze electroneutral transport of Ca^{2+} and Mg^{2+} across the mitochondrial inner membrane for the exchange of $2H^+$ (Pfeiffer et al., 1974). Figure 2 shows the effect of A23187 and RR on the membrane potential ($\Delta\Psi$) of N-RLM oxidizing succinate or glutamate-plus-malate. With succinate as a substrate (Figure 2, trace 1), N-RLM generated a stable steady-state $\Delta\Psi$, and the addition of 0.5 μM A23187 caused a sharp additional increase in $\Delta\Psi$. The increase in $\Delta\Psi$ was stable for several minutes. The addition of 5 μM RR to the mitochondria after A23187 caused a sharp decline in $\Delta\Psi$ to the level measured prior to the ionophore, followed by a slow decline in $\Delta\Psi$ (Figure 2, trace 1). This effect can be accounted for by a transformation of the ΔpH of the protonmotive force into $\Delta\Psi$ by electroneutral $Me^{2+}/2H^+$ exchange. This is consistent with the findings of Rottenberg and Scarpa (1974) who proposed a similar effect for nigericin, which catalyzes the electroneutral exchange of K^+ and H^+ ions. In contrast to succinate, mitochondria oxidizing glutamate plus malate generated a $\Delta\Psi$ which gradually declined with time (Figure 2, trace 2). The addition of A23187 to mitochondria oxidizing 20 mM glutamate plus 2 mM malate also caused a substantial (about 10 mV) increase in the $\Delta\Psi$ value and also decreased the rate of the $\Delta\Psi$ decline (Figure 2, trace 2). With α-ketoglutarate and pyruvate as substrates, A23187 caused a collapse of $\Delta\Psi$, evidently due to a severe inhibition of the corresponding dehydrogenases (not shown). Taken together, the increase in respiration induced by A23187 and RR could not be due to a direct uncoupling since their effects on state 4 were different depending upon the substrate used, and in most, but not all, cases could be reversed by the addition of Mg^{2+}. Hence this observation could be better explained by the depletion of mitochondrial Mg^{2+} and/or Ca^{2+} and the consequent effect on dehydrogenases.

Effects of A23187 and RR on the Redox State of Mitochondrial Pyridine Nucleotides. To estimate whether the inhibition by A23187 of state 4 respiration and the collapse of $\Delta\Psi$ with α-ketoglutarate and pyruvate as substrates were due to inhibition of dehydrogenases, the fluorescence of mitochondrial pyridine nucleotides was measured. These measurements were also essential in view of the recent observation (Panov & Scaduto, 1996), that in rat heart mitochondria there is a dissociation between the membrane potential value and the redox state of the mitochondrial pyridine nucleotides.

Figure 3 shows the pattern of the mitochondrial NAD(P)H fluorescence in the presence and absence of ionophore A23187. We observed that, with succinate as a substrate, the addition of A23187 did not cause any substantial changes in the level of NAD(P)H (Figure 3, traces 1 and 2), despite a significant increase in $\Delta\Psi$ (Figure 2). This is consistent with the observation that during state 4 oxidation of succinate (in the absence of rotenone), mitochondria have the highest level of NADH (Panov & Scaduto, 1996). With glutamate-plus-malate as substrates, the control liver mitochondria do not maintain a stable steady-state level of NAD(P)H; rather, it gradually declined over time (Figure 3, trace 3). A similar pattern of mitochondrial NAD(P)H was observed in rat heart mitochondria oxidizing glutamate-plus-malate (Panov & Scaduto, 1996). In the presence of A23187, the level of

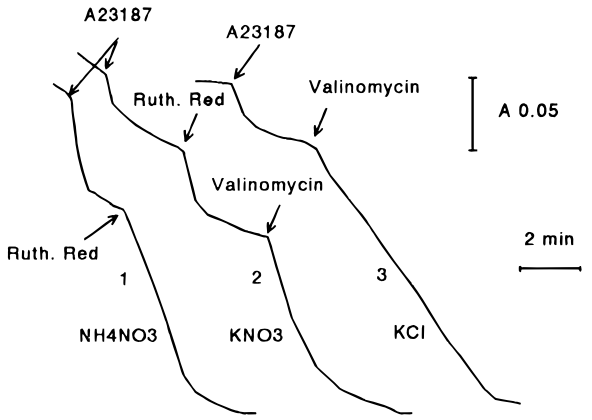

FIGURE 4: Effects of A23187 and ruthenium red on swelling of rat liver mitochondria. Incubation conditions: trace 1, 110 mM NH_4NO_3; trace 2, 110 mM KNO_3; trace 3, 125 mM KCl. All media contained 10 mM MOPS, pH 7.2; 0.5 mg of N-RLM/3 mL, 2 μg of rotenone/mg of RLM.

NAD(P)H fluorescence slightly increased, and the rate of NAD(P)H decline was slower than without the ionophore (Figure 3, trace 4). With pyruvate as a substrate, N-RLM generate a stable steady-state level of NAD(P)H (Figure 3, trace 5). In the presence of A23187, mitochondrial NAD(P)H was very low (Figure 3, trace 6), suggesting an inhibition of the pyruvate dehydrogenase complex activity. Fluorescence of the mitochondrial pyridine nucleotides with 10 mM α-ketoglutarate could not be measured because of strong quenching of the fluorescence by large concentrations of α-ketoglutarate.

Effects of A23187 and RR on Mitochondrial Permeability to H^+ and K^+. The next series of experiments was undertaken to attempt to explain the unanticipated uncoupling effect of RR in mitochondria treated with A23187. The data presented above indicate that the effects of Ca^{2+} and Mg^{2+} depletion by ionophore A23187 on mitochondrial respiration strongly depend on the substrate used. Since A23187 alone increased the inner membrane $\Delta\Psi$ with succinate and glutamate-plus-malate, whereas the combined action of A23187 and RR decreased mitochondrial $\Delta\Psi$ and increased state 4 respiration, one can assume that the two compounds together might increase the inner membrane permeability to cations (Kapus et al., 1990). A qualitative technique to study permeability of the inner membrane to H^+ and K^+ is to monitor changes in absorbance which, under most conditions, relate to changes in matrix volume (Beavis et al., 1985).

It has long been recognized that mitochondrial Mg^{2+} regulates the permeability of the inner mitochondrial membrane to monovalent cations [for review see Jung and Brierley (1986) and Brierley (1976)]. Kapus et al. (1990) have provided evidence that RR affects the electrogenic Na^+ or K^+ fluxes in Mg^{2+}-depleted rat liver mitochondria treated with EDTA or with EDTA-plus-A23187. These authors also suggested that Mg^{2+} is necessary for maintaining the Ca^{2+} selectivity of the Ca^{2+} uniporter, and that alterations in the mitochondrial Mg^{2+} content could induce the uniporter to operate specifically as a channel for monovalent cations (Kapus et al., 1990). To test this hypothesis, and to elucidate the mechanism by which A23187-plus-RR uncouples state 4 respiration, we investigated the effects of A23187 and RR on mitochondrial permeability for K^+ and H^+ ions.

Neither energized nor de-energized N-RLM swelled in a KCl medium (Figure 4, trace 3). The addition of 0.5 μM A23187 to de-energized N-RLM caused a rapid low-amplitude swelling of the mitochondria. The subsequent addition of RR after A23187 induced swelling of the mitochondria only if 1 mM EGTA was also present.

Using isotonic KNO_3 medium (Figure 4, trace 2), when the rate of osmotic swelling of the de-energized mitochondria depended on permeability of the inner membrane to K^+ ions (Mitchell & Moyle, 1968), N-RLM displayed a slow rate of swelling. The addition of 0.5 μM A23187 caused a rapid low-amplitude swelling of the mitochondria. At the new steady-state volume, the rate of mitochondrial swelling was almost the same as before the addition of A23187. The addition of 5 μM RR after the ionophore caused swelling of the mitochondria in the KNO_3 medium to a new steady-state volume. A further large-scale swelling of N-RLM could be initiated by adding 0.2 μM valinomycin (see Figure 4, trace 2). In an isotonic NH_4NO_3 medium, the rate of osmotic swelling of the de-energized mitochondria depends on permeability of the inner membrane for protons (Mitchell & Moyle, 1968). Figure 4 (trace 1) shows that, qualitatively, the effects of A23187 and RR on mitochondrial swelling were similar to those observed with the mitochondria incubated in a KNO_3 medium.

Thus, the addition of RR to the Ca^{2+} and Mg^{2+} depleted mitochondria increases the inner membrane permeability to small cations, K^+ and H^+, as evidenced by the data of Figure 4, and to Na^+, as was shown by Kapus et al. (1990).

DISCUSSION

The purpose of this work was to reassess the role of endogenous mitochondrial Mg^{2+} as a potential regulator of mitochondrial dehydrogenase activity and therefore of cellular respiration. While a study with similar intent was published nearly 25 years ago (Reid & Lardy, 1972), the results of this study were skewed by the unphysiologically high concentration of endogenous Ca^{2+} present in the mitochondria. Because of these conditions, this previous study concluded that only endogenous Ca^{2+} and not Mg^{2+} modulated activity of dehydrogenases. In the current study, isolation conditions leading to different endogenous divalent cation contents were standardized and the cation content and respiration were measured. Additionally, various protocols using the divalent cation ionophore A23187 and the mitochondrial uniporter inhibitor RR were used to effectively produce a mitochondrial cation depletion. The data presented in Table 1 clearly demonstrate that isolation of the liver mitochondria in the presence of EDTA and EGTA resulted in an almost 6-fold decrease in mitochondrial Ca^{2+} content. It may be suggested that excessive mitochondrial Ca^{2+} is a result of uptake by mitochondria of Ca^{2+} released during tissue homogenization. On the basis of the data presented by Somlyo et al. (1978), one can also assume that relatively high mitochondrial Ca^{2+} content occurs when mitochondria are isolated, even in the presence of EGTA. This could reflect an inhomogenous population of isolated mitochondria, some of which, originating from initially damaged cells, may be heavily loaded with Ca^{2+}.

Table 2 shows that the effects of A23187 and A23187-plus-RR on the mitochondrial respiration depend both on the substrate type and the metabolic state of the mitochondria. Because A23187, in the presence of 1 mM EGTA, decreases the mitochondrial content of both Ca^{2+} and Mg^{2+},

the effect on mitochondrial respiration could be accounted for by different sensitivity of the dehydrogenases to both or either of the divalent cations.

Of all substrates studied, the oxidation of α-ketoglutarate or pyruvate is most inhibited by A23187 in all metabolic states. Our data indicate that in state 4 (see Table 2), A23187 induces almost 2-fold inhibition of respiration, and both mitochondrial membrane potential (not shown) and NAD(P)H content (Figure 3, trace 6) were also decreased. These data suggest that the major effect of the ionophore on the oxidation of α-ketoglutarate or pyruvate could result from inhibition of the corresponding dehydrogenase complexes and therefore the inability to maintain a normal mitochondrial membrane potential. The inability of 2 mM Mg^{2+} to restore the oxidation of these two substrates in all metabolic states in the mitochondria treated with A23187 or A23187-plus-RR, might be interpreted as an indication that the ionophore directly inhibits the activities of these dehydrogenases, as proposed by Reed and Lardy (1972), or, alternatively, that these dehydrogenases also require Ca^{2+} for activity. As both α-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes require Ca^{2+} *in vitro* for maximal activity (McCormack & Denton, 1979; McCormack et al., 1990), the inhibition could be explained by the fact that A23187-treated N-RLM contained 3.3-fold less Ca^{2+} (1.1 ± 0.05 nmol of Ca^{2+}/mg of protein) than control RLM (3.5 ± 0.2 nmol of Ca^{2+}/mg of protein).

In state 4, A23187 stimulated only oxidation of succinate (Table 2), whereas the combined effect of A23187 plus RR increased the oxidation of succinate, glutamate-plus-malate, and acetylcarnitine. This implies that the effects of the ionophore alone, and A23187-plus-RR differ in their mechanisms. Since the state 4 oxidation of succinate is stimulated by EDTA (Lund & Wiggins, 1990) and was also stimulated in the mitochondria isolated in the presence of EGTA and EDTA, when compared with mitochondria isolated without these chelators (see Table 1), the stimulatory effect of A23187 on succinate oxidation is likely to be related to Mg^{2+} depletion. Consistently, the stimulation of state 4 respiration is absent in the presence of Mg^{2+} in the medium. The particular sensitivity of succinate oxidation to even a relatively small loss of Mg^{2+} from the mitochondria places succinate dehydrogenase, or the respiratory complex II as a possible target for regulation by physiologically relevant variations in mitochondrial Mg^{2+} content.

Uncoupling by A23187 plus RR represents an effect which may involve depletion of larger amounts of Ca^{2+} or Mg^{2+}, since it can be observed only in the presence of EGTA. It may also suggest that the additional removal of either Ca^{2+} or Mg^{2+} from the high-affinity binding sites is involved in the uncoupling effect of the combined addition of A23187-plus-RR. This agrees with the observation of Jung and Brierley (1986) that increases in swelling of the heart mitochondria are related to the removal of a component of mitochondrial Mg^{2+} that is bound with high-affinity sites rather than to decreases in matrix-free [Mg^{2+}]. Since Mg^{2+} completely restores or prevents the uncoupling action of A23187-plus-RR, it is reasonable to assume that Mg^{2+} is responsible for the effect. From data on the effects of A23187-plus-RR on mitochondrial membrane potential (Figure 2) and permeability to H^+ and K^+ (Figure 4) it can be deduced that the uncoupling effect is secondary to the increased ion permeability and de-energization of the inner membrane.

The data presented in Table 2 show that there is a strong substrate specificity for the effects of A23187 on mitochondrial oxidative phosphorylation. A relatively small inhibition by A23187 of state 3 oxidation of succinate, or of glutamate-plus-malate, also argues against the idea that ATP synthase is the only mechanism by which Mg^{2+} controls oxidative phosphorylation.

The results of measurements of $\Delta\Psi$ (Figure 2) and mitochondrial swelling (Figure 4) in mitochondria treated with A23187 and RR allow several conclusions. First, the uncoupling effect of A23187 on mitochondria oxidizing succinate is not due to a decrease in the membrane potential. On the contrary, A23187 increases $\Delta\Psi$ in mitochondria oxidizing succinate or glutamate-plus-malate. Conceivably, the increase in $\Delta\Psi$ may be caused by the electroneutral exchange of Ca^{2+} and Mg^{2+} for $2H^+$ (Pfeiffer et al., 1974). Secondly, the decrease in $\Delta\Psi$ by RR in the A23187-treated mitochondria seems to be secondary to the increased membrane permeability and swelling of the mitochondria (Figure 4).

Data in the literature on the effects of Mg^{2+} on isolated dehydrogenases (Thomas et al., 1986; Walsh et al., 1976; Moreno-Sanchez et al., 1995) and the data reported here allow us to conclude that, in addition to the free Mg^{2+} concentration, changes in the bound Mg^{2+} may be of regulatory importance for the mitochondrial respiratory enzymes. Some of the enzymes, like succinate dehydrogenase (respiratory complex II), have relatively low affinity for Mg^{2+}, and may be activated by the removal of Mg^{2+} by EDTA (Lund & Wiggins, 1990). Other enzymes, like α-KGDHC and PDHC, have higher affinity for Mg^{2+} and become strongly inhibited after removal of this cation from its specific binding sites. High-affinity binding sites for Mg^{2+} or Ca^{2+} also control inner membrane permeability to small ions (Jung & Brierley, 1986). Because the removal of Mg^{2+} from some high-affinity binding sites can have deleterious effects on mitochondrial functions, it is conceivable that one of the reasons that a cell maintains high concentration of free Mg^{2+} is to prevent such changes. Bond et al. (1987) have shown that hormonal stimulation of the vascular smooth muscle caused a significant decrease of the cytoplasmic Mg^{2+} and an increase in the mitochondrial Mg^{2+} content without changes in the mitochondrial Ca^{2+}. Recently, we have shown that with isolated α-ketoglutarate Mg^{2+} significantly increased the effect of low concentration of Ca^{2+} on the enzyme's affinity for α-ketoglutarate (Panov & Scarpa, 1996). Thus, in view of the data presented by Bond et al. (1987) and Romani et al. (1991, 1993) it is conceivable that the hormonal-induced changes in the mitochondrial Mg^{2+} content could affect activities of the Ca^{2+}-dependent dehydrogenases by modifying the effects of Ca^{2+} already present in the mitochondria.

In conclusion, the data presented in this paper clearly demonstrate that mitochondrial Mg^{2+} has multiple effects, both direct and indirect, on the respiratory activity of mitochondria. Indirect effects of Mg^{2+} may be caused by changes in Mg^{2+} complexed with ATP and other metabolites (Veloso et al., 1973; Bedino & Testore, 1992), or in Mg^{2+}-dependent permeability of the inner membrane (Brierley, 1976). Direct effects of Mg^{2+} on mitochondrial respiration are evidently connected with its effects on mitochondrial

dehydrogenases. Succinate dehydrogenase (respiratory complex II) is very sensitive to changes in mitochondrial Mg^{2+} content. Other dehydrogenases, namely, α-ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex, are evidently subject to control by both Mg^{2+} and Ca^{2+} within the mitochondria, as suggested by the experiments on isolated enzymes (McCormack & Denton, 1979; Panov & Scarpa, 1996).

In view of the different effect of mitochondrial Mg^{2+}, it is difficult, without additional work, to predict to which effect and in which direction changes of mitochondrial Mg^{2+} will affect overall respiration *in vivo*. Yet, it has been shown that Mg^{2+} can rapidly change within mitochondria *in situ* (Romani et al., 1991, 1993), and this work clearly indicate that several dehydrogenases could be greatly affected by mitochondrial Mg^{2+}. Hence, variation in mitochondrial Mg^{2+} should be of great metabolic consequence not only for the control of respiration but also for several metabolic pathways affected by increased or decreased concentrations of citric acid cycle metabolites.

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