Effect of micromolar concentrations of manganese ions on calcium-ion cycling in rat liver mitochondria

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The effects of micromolar concentrations of Mn^{2+} on the rat liver mitochondrial Ca^{2+} cycle were investigated. It was found that the addition of Mn^{2+} to mitochondria which were cycling $^{45}Ca^{2+}$ led to a rapid dose-dependent decrease in the concentration of extramitochondrial $^{45}Ca^{2+}$ of about 1 nmol/mg of protein. The effect was complete within 30 s, was half-maximal with $10\mu M-Mn^{2+}$ and was observed in the presence of 3 mM- Mg^{2+} and 1 mM-ATP. It occurred over a broad range of incubation temperatures, pH and mitochondrial Ca^{2+} loads. It was not observed when either Mg^{2+} or phosphate was absent from the incubation medium, or in the presence of Ruthenium Red. These findings indicate that micromolar concentrations of Mn^{2+} stimulate the uptake of Ca^{2+} by rat liver mitochondria, and provide evidence for an interaction between Mg^{2+} and Mn^{2+} in the control of mitochondrial Ca^{2+} cycling.

Rat liver mitochondria are able to regulate finely the concentration of extramitochondrial Ca^{2+} , owing to the presence of pathways for Ca^{2+} uptake and efflux (Nicholls, 1978; Becker *et al.*, 1980). There is also evidence that alterations in rat liver mitochondrial Ca^{2+} fluxes are involved in the activation of glycogenolysis by α_1 -adrenergic agents (Blackmore *et al.*, 1979a,b; Babcock *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981; Berthon *et al.*, 1981; Kimura *et al.*, 1982).

Many factors induce the release of Ca^{2+} from mitochondria. These include phosphoenolpyruvate (Roos *et al.*, 1978), prostaglandins (Crompton *et al.*, 1978), increased oxidation of nicotinamide-adenine nucleotides (Lehninger *et al.*, 1978; Prpic & Bygrave, 1980; Wolkowicz & McMillin-Wood, 1980; Nicholls & Brand, 1980; Hofstetter *et al.*, 1981), cyclic AMP (Arshad & Holdsworth, 1980) and decreased extramitochondrial pH (Akerman, 1978; Tsokos *et al.*, 1980). Other agents alter the Ca^{2+} cycle of mitochondria such that they buffer Ca^{2+} at a higher extramitochondrial concentration. These include Mg^{2+} (Nicholls, 1978) and Na^+ (Nedergaard & Cannon, 1980; Haworth *et al.*, 1980; Hughes *et al.*, 1980; Heffron & Harris, 1981). However, little is known of agents which induce the uptake of Ca^{2+} by mitochondria which are cycling Ca^{2+} , such that there is a decrease in extramitochondrial Ca^{2+} . In this connection, we have

examined the potential of Mn^{2+} to regulate the mitochondrial Ca^{2+} cycle.

Mn^{2+} can be transported into rat liver mitochondria in an energy-dependent manner, probably via the Ca^{2+} carrier, at a rate much slower than Ca^{2+} (Chappell *et al.*, 1963; Ernster & Nordenbrand, 1967; Mela & Chance, 1968; Vainio *et al.*, 1970; Vinogradov & Scarpa, 1973; Case, 1975; Puskin *et al.*, 1976; Gunter *et al.*, 1978). It has been observed that Ca^{2+} is able to accelerate the transport of Mn^{2+} into mitochondria (Ernster & Nordenbrand, 1967; Mela & Chance, 1968; Vainio *et al.*, 1970; Vinogradov & Scarpa, 1973). However, to our knowledge, the possible role of Mn^{2+} as a regulator of mitochondrial Ca^{2+} fluxes has not been addressed.

Experimental

Isolation of mitochondria and measurement of Ca^{2+} and Mn^{2+} fluxes

'Heavy mitochondria' (i.e. those sedimented between 3800 and 26 000 g-min) were isolated from the livers of fed Sprague-Dawley rats (200–300 g), and the protein concentration was measured as described by Dehay *et al.* (1981). Mitochondrial $^{45}Ca^{2+}$ transport was routinely measured in a medium (final volume 2 ml) containing 120 mM-KCl, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-

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ethanesulphonic acid]/KOH, 5 mM-succinate (disodium salt), 2 mM- KH_2PO_4 , 3 mM- MgCl_2 and 2 mg of mitochondrial protein (pH 7.4 at 30°C) in a magnetically stirred water-jacketed incubation chamber. Unless otherwise indicated the concentration of $^{45}\text{Ca}^{2+}$ added as $^{45}\text{CaCO}_3$ (0.29 μCi) was 20 nmol/mg of protein. Other additions were made as described in the text and legends. Before any samples were removed, a pre-equilibration time of 5 min was allowed. Samples (100 μl) were removed in triplicate or quadruplicate and placed into tubes containing 100 μl of ice-cold 'quench medium' (Reed & Bygrave, 1974), consisting of 320 mM-KCl, 4 μM -Ruthenium Red and 1 mM-EGTA. The tubes were mixed rapidly and centrifuged for 1 min in an Eppendorf centrifuge (model 5412). A 100 μl portion of the supernatant was placed into plastic vials (Beckman Bantam vials), 5 ml of scintillant (ACS; Amersham) was added, and the radioactivity was counted. Extramitochondrial $^{45}\text{Ca}^{2+}$ was defined as that present in the supernatant. A similar procedure was used to estimate the total amount of $^{45}\text{Ca}^{2+}$ present in the incubation, except that the samples were not centrifuged. After removal of the samples for measurement of the initial extramitochondrial $^{45}\text{Ca}^{2+}$ concentration, MnCl_2 (or the other agents under test) was added, and at the desired time (2 min, except in the time-course experiments) 100 μl samples were removed in triplicate or quadruplicate and processed as above. In calculating the specific radioactivity of the $^{45}\text{Ca}^{2+}$ in the incubations, correction was made to include the endogenous Ca^{2+} .

Some comment is necessary on the use of $^{45}\text{Ca}^{2+}$ to monitor Ca^{2+} movements in the present study. Although several investigations of mitochondrial buffering of Ca^{2+} have used either a Ca^{2+} -selective electrode or a Ca^{2+} -sensitive dye such as arsenazo to measure the free Ca^{2+} concentration in the medium (Nicholls, 1978; Nicholls & Scott, 1980; Becker *et al.*, 1980; Coll *et al.*, 1982), these methods were not successful in the present study. This was because Mn^{2+} caused severe interference with the Ca^{2+} electrode and could bind strongly to arsenazo and antipyrilazo. In addition, both types of techniques suffered from a loss of sensitivity in the presence of millimolar amounts of Mg^{2+} , which is an important consideration when the observed effect of Mn^{2+} on extramitochondrial Ca^{2+} was only about 1 μM .

An alternative system for the measurement of free Ca^{2+} in the medium is the use of $^{45}\text{Ca}^{2+}$ in the presence of Ca^{2+} chelators (Nicholls, 1978; Barritt, 1981). However, in the present situation, this technique was not readily applicable, as the cations under test, e.g. Mn^{2+} and Sr^{2+} , bound to the chelators with affinities close to that of Ca^{2+} (Bartfai, 1979) and, more importantly, were transported into the mitochondria (Vainio *et al.*, 1970),

resulting in different concentrations of the ions initially and at equilibrium. Consequently, in order to avoid the problems associated with the measurement of free Ca^{2+} concentration, the technique of monitoring extramitochondrial $^{45}\text{Ca}^{2+}$ concentration was used. The value obtained for the extramitochondrial $^{45}\text{Ca}^{2+}$ concentration, i.e. 3–4 μM , is in good agreement with that obtained by Nicholls (1978), in the absence of added chelator, for the free Ca^{2+} concentration of the medium.

To monitor the transport of $^{54}\text{Mn}^{2+}$, the same procedure was used except that $^{40}\text{Ca}^{2+}$ was present in place of $^{45}\text{Ca}^{2+}$. $^{54}\text{MnCl}_2$ (0.9 Ci/nmol) was added at the concentration indicated in the text and legends, and 250 μl of water rather than scintillation fluid was added to each sample before counting of the samples for radioactivity in a Beckman Gamma 7000 counter.

All experiments were conducted with duplicate or triplicate incubations. To ensure reproducibility, each experiment was performed with at least two batches of mitochondria. The data are presented as representative experiments.

To measure the endogenous Ca^{2+} and Mn^{2+} associated with mitochondria, 1 ml samples (approx. 40 mg of protein/ml) were added to 1 ml of 0.6 M- HClO_4 containing 0.1% (w/v) LaCl_3 . The tubes were mixed, centrifuged at 500 g for 10 min, and the amount of Ca^{2+} or Mn^{2+} present in the supernatant was measured with a Perkin-Elmer 603 atomic-absorption spectrophotometer. The concentrations of endogenous Ca^{2+} and Mn^{2+} associated with the mitochondria were 6.5 ± 0.30 and 0.41 ± 0.02 nmol/mg of protein ($n = 15$, mean $\pm$ S.E.M.) respectively.

Measurement of apparent free Mn^{2+} concentration

Free Mg^{2+} , Mn^{2+} and ATP were measured by established procedures (Moe & Butler, 1972; Bartfai, 1979). Values for the association constants for MgATP and MnATP were taken from the literature (Bartfai, 1979). The chosen values were $K_{\text{Mg}} = 5.75 \times 10^{-5} \text{ M}$ and $K_{\text{Mn}} = 1.33 \times 10^{-5} \text{ M}$. It was assumed that the amount of extramitochondrial Ca^{2+} (i.e. 1 μM) and the chelating species succinate and phosphate would not significantly alter the values obtained.

Materials

$^{45}\text{Ca}^{2+}$ and $^{54}\text{Mn}^{2+}$ were obtained from New England Nuclear. Na_2ATP was obtained from Boehringer Mannheim. Oligomycin was purchased from Calbiochem. Atractyloside (sodium salt) was purchased from Sigma. The bivalent metal ions were purchased as their chloride salts from Fisher. Other materials were obtained from sources noted previously (Dehay *et al.*, 1981).

Results

Effect of Mn²⁺ on mitochondrial Ca²⁺ cycling

The addition of 40 μ M-Mn²⁺ to mitochondria incubated at 30°C in a medium consisting of 120 mM-KCl, 10 mM-Hepes/KOH, pH 7.4, 5 mM-succinate (disodium salt), 3 mM-MgCl₂, 2 mM-KH₂PO₄ and 20 nmol of ⁴⁵Ca²⁺/mg of protein (added ⁴⁵Ca²⁺) led to a rapid decrease in extramitochondrial ⁴⁵Ca²⁺ of about 1.1 nmol/mg of protein (Fig. 1). This effect was evident within 15 s and required about 30 s for full development. It was maintained for at least 8 min, i.e. the longest time after Mn²⁺ addition determined (results not shown). The effect of Mn²⁺ was dose-dependent, with consistent effects being observed with 2.5 μ M added Mn²⁺. A half-maximal effect was seen at about 10 μ M and a maximal effect at about 80 μ M added Mn²⁺ (results not shown). The

Fig. 1. Time course of Mn²⁺ effect on extramitochondrial ⁴⁵Ca²⁺ concentrations

Mitochondria were isolated and incubated as described in the Experimental section. After an equilibration period of 5 min, samples were removed to estimate the extramitochondrial ⁴⁵Ca²⁺ concentration. At the time indicated as $t = 0$, 40 μ M-MnCl₂ (●) or water (O) was added. Samples were then removed to measure extramitochondrial ⁴⁵Ca²⁺ concentration at the times indicated.

addition of 1 μ M-Ruthenium Red, an inhibitor of mitochondrial Ca²⁺ uptake (Moore, 1971; Vasington *et al.*, 1972; Reed & Bygrave, 1974), resulted in an increase in extramitochondrial ⁴⁵Ca²⁺ concentration (Fig. 2). Under these conditions, the addition of 40 μ M-Mn²⁺ was without effect on the concentration of extramitochondrial ⁴⁵Ca²⁺ (Fig. 2). To ensure that the observed effect of Mn²⁺ on mitochondrial Ca²⁺ cycling was not due merely to the use of ⁴⁵Ca²⁺, experiments were conducted in which the amount of ⁴⁰Ca²⁺ associated with the mitochondrial pellet was measured by atomic-absorption spectroscopy. The values obtained were essentially identical with those obtained in parallel experiments conducted with ⁴⁵Ca²⁺ (results not shown).

To test the specificity of Mn²⁺ and also to rule out the involvement of the counter-ion, i.e. Cl⁻, other bivalent trace-metal ions were tested (Table 1). Of those tested, Mn²⁺, Sr²⁺ and to a lesser extent Fe²⁺ stimulated the uptake of ⁴⁵Ca²⁺. As might be predicted from their general toxic effects (Mills, 1981), Cu²⁺, Zn²⁺, Cd²⁺ and Hg²⁺ caused large releases of ⁴⁵Ca²⁺ (Table 1).

We wished to know the extent of Mn²⁺ uptake by the mitochondria under the present conditions. The data in Fig. 3(a) show that ⁵⁴Mn²⁺ was taken up by the mitochondria and that both the apparent initial rate of uptake and the amount taken up in 2 min increased linearly relative to the amount of added ⁵⁴Mn²⁺ (Fig. 3b).

Fig. 2. Effect of Ruthenium Red on the Mn²⁺ effect on extramitochondrial ⁴⁵Ca²⁺ concentration

The experimental conditions were the same as in Fig. 1, except that at $t = 0$, 1 μ M-Ruthenium Red was added, and at $t = 1$ min, water (O) or 40 μ M-MnCl₂ (●) was added.

It has become apparent from the work of Nicholls and others (Nicholls & Scott, 1980; Nicholls & Brand, 1980; Hofstetter *et al.*, 1981) that, in

Table 1. *Effect of bivalent cations on extramitochondrial ⁴⁵Ca²⁺ concentrations*

Mitochondria were isolated and incubated as described in the Experimental section. The bivalent metal ions were added as their Cl⁻ salts, except for Fe²⁺, which was the SO₄²⁻ salt. The data are the means ± S.E.M. for four samples.

Bivalent cation (40 μM)	Extramitochondrial ⁴⁵ Ca ²⁺ (nmol/mg of protein)	
	Initial value	2 min after addition
— (water)	3.8 ± 0.1	4.2 ± 0.1
Mg ²⁺	3.8 ± 0.1	4.0 ± 0.1
Mn ²⁺	4.1 ± 0.1	3.0 ± 0.1
Fe ²⁺	4.0 ± 0.1	3.6 ± 0.1
Co ²⁺	3.8 ± 0.04	5.6 ± 0.2
Ni ²⁺	3.8 ± 0.2	4.9 ± 0.2
Cu ²⁺	4.0 ± 0.1	27.3 ± 0.3
Zn ²⁺	4.0 ± 0.1	24.6 ± 0.8
Sn ²⁺	4.4 ± 0.1	5.0 ± 0.1
Cd ²⁺	4.9 ± 0.1	26.0 ± 0.1
Ba ²⁺	3.8 ± 0.1	4.1 ± 0.1
Hg ²⁺	3.8 ± 0.1	26.8 ± 0.2
Sr ²⁺	4.2 ± 0.03	3.2 ± 0.1

assessing potential controllers of mitochondrial Ca²⁺ cycling, consideration should be given to the concentration of ATP in the incubation medium. ATP is present in the cytoplasm in millimolar amounts (Akerboom *et al.*, 1978) and appears to exert a general protective effect on isolated mitochondria: for example, it inhibits the release of Ca²⁺ from mitochondria induced by phosphoenolpyruvate (Roos *et al.*, 1978), acetoacetate (Nicholls & Brand, 1980) and hydroperoxide (Hofstetter *et al.*, 1981). Consequently, it was important to assess the effect of Mn²⁺ on mitochondrial Ca²⁺ cycling in the presence of ATP and to determine whether the characteristics of Mn²⁺ transport into mitochondria were unchanged by the presence of this compound.

Initial experiments indicated that ATP caused considerable inhibition of the effect of Mn²⁺ on mitochondrial ⁴⁵Ca²⁺ uptake (results not shown). However, this approach did not take into account the fact that the association constant for MnATP is approximately 5 times that for MgATP (Bartfai, 1979). Consequently, it was necessary to consider (as described in the Experimental section) the relationship between the amount of total Mn²⁺ added and the apparent free Mn²⁺ concentration. The data in Fig. 4(a) show that the effect of Mn²⁺ on mitochondrial ⁴⁵Ca²⁺ uptake was similar whether

Fig. 3. *Uptake of ⁵⁴Mn²⁺ by mitochondria*

The experimental conditions were the same as in Fig. 1, except that ⁴⁰Ca²⁺ was present instead of ⁴⁵Ca²⁺. In (a), ⁵⁴MnCl₂ was added and samples were removed at the times indicated and the amount of ⁵⁴Mn²⁺ associated with the mitochondria was determined. The values in parentheses represent the concentrations of added ⁵⁴MnCl₂, in nmol/mg of protein. In (b), the apparent 'initial' rates (▲), i.e. amount of ⁵⁴Mn²⁺ taken up in the first 15 s, and the total amount taken up in 2 min (●) are plotted as a function of added ⁵⁴Mn²⁺. The lines are derived by linear regression analysis.

ATP was omitted or present at 1 mM when correction was made for the binding of Mn²⁺ to ATP. The apparent initial rates of ⁵⁴Mn²⁺ uptake were also similar in the presence or absence of ATP (Fig. 4b), although at later times more ⁵⁴Mn²⁺ was taken up in the presence of ATP, owing to the higher amounts of total ⁵⁴Mn²⁺ added under this condition. It is noteworthy that, with 3 mM-MgCl₂ and 1 mM-Na₂ATP, it was necessary to add approximately 3 times more Mn²⁺ than that required in the absence of ATP in order to obtain the same apparent free Mn²⁺ concentration (legend, Fig. 4).

Effect of temperature, pH and Ca²⁺ load

The effect of Mn²⁺ on mitochondrial ⁴⁵Ca²⁺ cycling was observed at all pH values from 6.6 to 8.2 (Fig. 5a). At higher pH values, the initial extramitochondrial ⁴⁵Ca²⁺ concentration was lower than that observed at lower pH, presumably owing to an increase in activity of the Ca²⁺ uniport at more alkaline pH (Heaton & Nicholls, 1976).

Increasing the temperature from 1° to 30°C led to increased values for extramitochondrial ⁴⁵Ca²⁺

(Fig. 5b). At temperatures below 4°C, addition of Mn²⁺ was without effect, but as the temperature was raised Mn²⁺ became increasingly effective. The observation that extramitochondrial ⁴⁵Ca²⁺ was less at lower temperatures may indicate that under these conditions the efflux pathway is not fully operative, or alternatively may reflect less damage to mitochondria at lower incubation temperatures.

As shown in Fig. 5(c), increasing the load of added ⁴⁵Ca²⁺ led to increased values for extramitochondrial ⁴⁵Ca²⁺. The addition of Mn²⁺ resulted in a decreased value for extramitochondrial ⁴⁵Ca²⁺ at all ⁴⁵Ca²⁺ loads tested, i.e. from zero added Ca²⁺ (i.e. tracer ⁴⁵Ca²⁺ added) up to 100 nmol of added ⁴⁵Ca²⁺/mg of protein.

Effect of omission of exogenous phosphate

It is well known that addition of phosphate results in an increased rate and capacity for mitochondrial Ca²⁺ uptake (Rossi & Lehninger, 1964), and it has been proposed that transport of Ca²⁺ and of phosphate are closely related in energized liver mitochondria (Rugulo *et al.*, 1981). Consequently

Fig. 4. Effect of Mn²⁺ on extramitochondrial ⁴⁵Ca²⁺ concentration and the uptake of ⁵⁴Mn²⁺ by mitochondria in the presence of ATP

In (a), the experimental conditions were the same as in Fig. 1, except that 50 μM-atractyloside and oligomycin (4 μg/mg of protein) were present to prevent transport and hydrolysis of ATP. The amount of Mn²⁺ added was adjusted such that the apparent free Mn²⁺ concentrations were the same in the absence (○) and presence of 1 mM-Na₂ATP (●). In the presence of 1 mM-Na₂ATP, the total amounts of Mn²⁺ needed to give the corresponding calculated concentrations of free Mn²⁺ were: 80 μM total = 27.4 μM free; 160 μM total = 57.6 μM free; 320 μM total = 126 μM free. In (b), the conditions were the same as in (a), except that ⁴⁰Ca²⁺ was present in place of ⁴⁵Ca²⁺. Open symbols represent incubations in the absence of ATP, so that total Mn²⁺ added approximates to the free Mn²⁺ concentration. Closed symbols represent incubations in the presence of 1 mM-Na₂ATP, so that free Mn²⁺ must be calculated as described in the Experimental section. The adjustments for the binding of Mn²⁺ to ATP were the same as in (a). Free Mn²⁺ was as follows: ○, ●, 27.4 μM; △, ▲, 57.6 μM; □, ■, 126 μM.

Fig. 5. Effect of incubation pH, temperature and mitochondrial $^{45}\text{Ca}^{2+}$ load

In (a), the experimental conditions were the same as in Fig. 1, except that the pH was varied as shown. The values for extramitochondrial $^{45}\text{Ca}^{2+}$ are those present initially (Δ) or 2 min after the addition of $40\mu\text{M-MnCl}_2$ ($\blacktriangle$). In (b), the experimental conditions were the same as in (a), except that the temperature of the incubation was varied as shown. In (c), the experimental conditions were the same as in (a), except that the mitochondrial $^{45}\text{Ca}^{2+}$ load was varied as shown. For the lowest $^{45}\text{Ca}^{2+}$ load, only tracer $^{45}\text{Ca}^{2+}$ ($2.3\mu\text{Ci}$) was added. $^{45}\text{Ca}^{2+}$ load refers to the total added $^{45}\text{Ca}^{2+}$.

the results of omission of added phosphate on the effect of Mn^{2+} on mitochondrial $^{45}\text{Ca}^{2+}$ cycling were investigated. Omission of phosphate completely inhibited the effect of Mn^{2+} on mitochondrial $^{45}\text{Ca}^{2+}$ cycling (Fig. 6a). The effect was partially restored by $100\mu\text{M-phosphate}$ and fully restored by 2mM-phosphate (Fig. 6b). The observation that varying the concentration of phosphate had little effect on the initial (i.e. in the absence of Mn^{2+}) concentration of extramitochondrial $^{45}\text{Ca}^{2+}$ requires comment. Although it is well established that phosphate increases the rate and capacity for mitochondrial Ca^{2+} uptake, it has also been shown that at Ca^{2+} loads of 50nmol/mg of protein or less, phosphate or acetate does not significantly alter the steady-state pCa^{2+} value, relative to that obtained in the absence of permeant anion (Nicholls, 1978). The present data, in which the concentration of added $^{45}\text{Ca}^{2+}$ was less than 30nmol/mg of protein, are thus in agreement with the observation of Nicholls (1978). The effect of phosphate appeared to be a specific effect rather than a general counter-anion effect, since when $2\text{mM-potassium acetate}$ was substituted for phosphate no effect of Mn^{2+} on mitochondrial $^{45}\text{Ca}^{2+}$ cycling was observed (results not shown).

The data in Fig. 7 show the effect of phosphate omission on the uptake of $^{54}\text{Mn}^{2+}$. Although there was a significant decrease in both the rate and extent of $^{54}\text{Mn}^{2+}$ uptake in the absence of added phosphate compared with that in the presence of 2mM-phosphate , the uptake of $^{54}\text{Mn}^{2+}$ still proceeded at appreciable rates in the absence of the anion.

Effect of Mg^{2+}

Mg^{2+} is known to decrease the initial rate of Ca^{2+} uptake by liver mitochondria and to increase the degree of sigmoidicity in the curve relating the initial rate of Ca^{2+} uptake to the external free Ca^{2+} concentration (Hutson *et al.*, 1976). Mg^{2+} is also known to increase the value at which mitochondria will buffer extramitochondrial free Ca^{2+} concentrations (Nicholls, 1978). Consequently, a possible explanation for the effects of Mn^{2+} on mitochondrial $^{45}\text{Ca}^{2+}$ cycling was that Mn^{2+} antagonized the inhibitory effects of Mg^{2+} on the activity of the Ca^{2+} uniport. This possibility was tested. The data in Fig. 8(a) show the effect of varying the Mg^{2+} concentration from 0 to 3mM on mitochondrial $^{45}\text{Ca}^{2+}$ cycling. In either the absence of added Mg^{2+} or in the presence of $100\mu\text{M-Mg}^{2+}$, Mn^{2+} addition caused no decrease in the value for extramitochondrial $^{45}\text{Ca}^{2+}$, whereas at higher concentrations a decrease was observed. The values for extramitochondrial $^{45}\text{Ca}^{2+}$ concentration in the presence of 0.5mM- , 1mM- or 3mM-Mg^{2+} and $40\mu\text{M-Mn}^{2+}$ were less than that observed in the absence of added Mg^{2+} and Mn^{2+} . Consequently it is not likely that the actions

Fig. 6. Effect of variable phosphate concentrations on the Mn²⁺ effect

In (a), the experimental conditions were the same as Fig. 1, except that KH₂PO₄ was omitted. In (b), the experimental conditions were the same as in (a), except that the concentration of KH₂PO₄ was varied as shown. The values for extramitochondrial ⁴⁵Ca²⁺ are those present initially (O) or 2 min after the addition of 40 μM-MnCl₂ (●).

Fig. 7. Effect of phosphate absence on the uptake of ⁵⁴Mn²⁺

The experimental conditions were the same as Fig. 3, except that KH₂PO₄ was either absent (O) or present at 2 mM (●) and the concentration of ⁵⁴Mn²⁺ was 40 μM, i.e. 40 nmol/mg of protein.

of Mn²⁺ are due merely to an antagonism of the effect of Mg²⁺ on the Ca²⁺ uniport.

Fig. 8(b) shows the effect of varying the Mg²⁺ concentration on the uptake of ⁵⁴Mn²⁺. As previously reported (Vainio *et al.*, 1970), low amounts of Mg²⁺ facilitated the transport of Mn²⁺ by the mitochondria. The rate of ⁵⁴Mn²⁺ uptake in the presence of 3 mM-Mg²⁺ shown in Fig. 8(b) was significantly less than that observed in the experiments of Figs. 3 or 7, because no exogenous Ca²⁺ was added. This was done to minimize the damage to

mitochondria that is known to occur when these organelles are incubated in the presence of even modest amounts of added Ca²⁺ and phosphate in the absence of added Mg²⁺ (Toninello *et al.*, 1982). Consequently, as these experiments were designed to test the effects of variable Mg²⁺ concentrations, only tracer amounts of ⁴⁵Ca²⁺ were added (Fig. 8a) or no Ca²⁺ was added (Fig. 8b). The observation that the transport of ⁵⁴Mn²⁺ by mitochondria is decreased in the absence of Ca²⁺ confirms previous findings (Vinogradov & Scarpa, 1973).

These results suggest that the effect of Mn²⁺ on mitochondrial ⁴⁵Ca²⁺ cycling is closely linked to the transport of Mn²⁺. To test this hypothesis further, the effects of a broader range of Mg²⁺ concentrations were examined. As shown in Fig. 9(a), increasing Mg²⁺ from 3 mM to 10 mM did not increase the rate of ⁵⁴Mn²⁺ transport by the mitochondria, but 30 mM-Mg²⁺ decreased the rate to a value very similar to that obtained in the absence of added Mg²⁺. These data agree with the 'bell-shaped' curve for the initial rate of Mn²⁺ transport as a function of added Mg²⁺ concentration reported by Vainio *et al.* (1970). In contrast with the effect of higher concentrations of Mg²⁺ on the uptake of ⁵⁴Mn²⁺ by the mitochondria, the effect of Mn²⁺ on the extramitochondrial ⁴⁵Ca²⁺ concentration was maintained as the concentration of Mg²⁺ was increased (Fig. 9b). These data indicate that the effects of Mn²⁺ on mitochondrial Ca²⁺ cycling can be observed under conditions (i.e. 30 mM-Mg²⁺) in which the transport of ⁵⁴Mn²⁺ is severely inhibited.

Effect of Mg²⁺ and phosphate on the effect of Sr²⁺ on mitochondrial Ca²⁺ cycling

As was shown in Table 1, addition of Sr²⁺ to the mitochondrial incubation also decreased the extra-

Fig. 8. Effect of variable Mg^{2+} concentrations on the Mn^{2+} effect on extramitochondrial $^{45}\text{Ca}^{2+}$ concentration and on the uptake of $^{54}\text{Mn}^{2+}$.

Mitochondria were incubated in a medium (final volume 2 ml) containing 120 mM-KCl, 10 mM-Hepes/KOH, 5 mM-succinate (disodium salt), 2 mM- KH_2PO_4 and 2 mg mitochondrial protein (pH 7.4 at 30°C). In (a), the concentration of MgCl_2 was varied as shown and only tracer $^{45}\text{Ca}^{2+}$ ($2.3\text{ }\mu\text{Ci}$) was added. The values for extramitochondrial $^{45}\text{Ca}^{2+}$ are those present initially (O) or 2 min after the addition of $40\text{ }\mu\text{M}$ MnCl_2 (●). In (b), the concentration (mM) of MgCl_2 was varied as shown in parentheses, the concentration of $^{54}\text{Mn}^{2+}$ was $40\text{ }\mu\text{M}$, i.e. 40 nmol/mg of protein, and there was no added Ca^{2+} .

Fig. 9. Effect of higher Mg^{2+} concentrations on the uptake of $^{54}\text{Mn}^{2+}$ and on the Mn^{2+} effect on extramitochondrial $^{45}\text{Ca}^{2+}$ concentration.

In (a), the experimental conditions were the same as in Fig. 8(b). The concentration of MgCl_2 (mM) was varied as shown in parentheses. In (b), the experimental conditions were the same as in Fig. 8(a). The concentration of MgCl_2 was varied as shown.

mitochondrial $^{45}\text{Ca}^{2+}$ concentration. The effect of Sr^{2+} on mitochondrial $^{45}\text{Ca}^{2+}$ cycling was similar to that of Mn^{2+} in that very little effect was observed in the absence of added phosphate, and acetate would not substitute for phosphate (results not shown). However, in contrast with the effect of Mn^{2+} , the effect of Sr^{2+} did not require added Mg^{2+} (results not shown).

Discussion

The elegant studies by Vinogradov & Scarpa (1973) of the initial rate of transport of Ca^{2+} and Mn^{2+} in rat liver mitochondria provided strong evidence for interaction between the transport of Ca^{2+} and Mn^{2+} . They observed that preloading of mitochondria with small amounts of Ca^{2+} increased

the initial rate of Mn²⁺ accumulation and changed the shape of the curve relating the rate of Mn²⁺ transport to the concentration of added Mn²⁺. Similar results had been observed by other workers (Ernster & Nordenbrand, 1967; Mela & Chance, 1968; Vainio *et al.*, 1970).

Vinogradov & Scarpa (1973) observed a linear double-reciprocal plot when Ca²⁺ uptake was plotted against the square of [Ca²⁺] in the medium, with a Hill coefficient approaching 2, which they interpreted as indicating that at least 2 molecules of Ca²⁺ must be bound to the transport system in order to have Ca²⁺ transported at significant rates. They proposed a model to account for the co-operativity observed in the transport of Ca²⁺ and between the transport of Ca²⁺ and Mn²⁺. Briefly, this model can be described as follows. The ion-transport system located within the mitochondrial inner membrane is able to bind two Ca²⁺ ions, and the resulting complex moves efficiently across the membrane. By contrast, the mobility of the complex with only one Ca²⁺ ion is very slow. A similar mechanism was proposed for the transport of Mn²⁺, but the affinity of the complex and the overall translocation rate were postulated to be lower. However, the mobility of the mixed complex of one Mn²⁺ and one Ca²⁺ ion was proposed to be faster, so that the presence of Ca²⁺ ions increased the transport of Mn²⁺.

In contrast, in the present study, the effect of Mn²⁺ on the rate of Ca²⁺ uptake by mitochondria appears not to be due to the co-transport of Ca²⁺ and Mn²⁺, for several reasons. Firstly, in the presence of 30 mM-Mg²⁺ the transport of ⁵⁴Mn²⁺ is retarded to that obtained in the absence of Mg²⁺. However, the effect of Mn²⁺ on the concentration of extramitochondrial ⁴⁵Ca²⁺ is observed in the presence of 30 mM-Mg²⁺, but not in the absence of Mg²⁺. Secondly, in the absence of phosphate, no effect of Mn²⁺ is observed on the extramitochondrial ⁴⁵Ca²⁺ concentration, even though transport of ⁵⁴Mn²⁺ is still able to proceed. In fact, the degree of ⁵⁴Mn²⁺ transport in the absence of added phosphate (see Fig. 7) is as great as that in the presence of 2 mM-phosphate, 3 mM-Mg²⁺ and the absence of added Ca²⁺ (see Fig. 8b). These are the conditions under which the full effect of Mn²⁺ on extramitochondrial ⁴⁵Ca²⁺ concentration is observed (see Fig. 8a). Thirdly, although the effect of Mn²⁺ on extramitochondrial ⁴⁵Ca²⁺ concentration is complete within 30 s and shows saturation with respect to the amount of Mn²⁺ added, the transport of Mn²⁺ is still progressing at 2 min, and both the rate and extent of Mn²⁺ transport increase linearly over the concentration range of Mn²⁺ tested (see Fig. 3).

The effect of Mn²⁺ on Ca²⁺ uptake observed in the present studies may be explained by the presence of a high-affinity Mn²⁺-binding site which is separate from the site for Mn²⁺ transport on the carrier.

In the presence of Mg²⁺, Mn²⁺ is able to bind to this site, which in turn increases the rate of Ca²⁺ transport, possibly by conferring a more active configuration. This process does not occur when Mg²⁺ is absent, but does occur even when Mg²⁺ is present at 30 mM. However, it should be kept in mind that the present data do not exclude a possible interaction between possible effects of Mg²⁺ and Mn²⁺ on the efflux component of the mitochondrial Ca²⁺ cycle. The transport of Mn²⁺ would be proposed to take place at a separate site. In the presence of low Mg²⁺, either the binding and/or transport of Mn²⁺ would be enhanced. Mg²⁺ at 30 mM may be able to compete with the binding of Mn²⁺ to the transport site and thus inhibit Mn²⁺ uptake. Under the present incubation conditions little transport of Mg²⁺ would be expected (Kun, 1976). Several mechanisms are feasible for the ability of Mg²⁺ to enhance the binding and/or transport of Mn²⁺. The binding of Mg²⁺ may cause a conformational change in the carrier. Alternatively, as has been pointed out by Vainio *et al.* (1970), Mn²⁺ has a larger hydrated-ion radius than Mg²⁺, and Mg²⁺ may act by 'opening' the membrane so that the hydrated Mn²⁺ ion can better reach its site(s) on the carrier.

In contrast with the effect of Mn²⁺ on the mitochondrial Ca²⁺ cycle, Sr²⁺ does not require the presence of added Mg²⁺ in order to exert its effect. This may indicate that the binding of Sr²⁺ to the carrier takes place at a different site from that (those) of Mn²⁺. The concept that the binding of Sr²⁺ and Mn²⁺ takes place at different sites on the carrier has been previously proposed (Reynafarje & Lehninger, 1969; Vainio *et al.*, 1970).

The present data provide evidence for a dual role for Mg²⁺ in the control of mitochondrial Ca²⁺ cycling. An inhibitory effect of Mg²⁺ on the initial rate of Ca²⁺ uptake (Hutson *et al.*, 1976) and an effect of Mg²⁺ to increase the value for the 'set point' for mitochondrial Ca²⁺ buffering (Nicholls, 1978) are well described. However, the present study clearly shows that Mg²⁺ is necessary to potentiate the effect of Mn²⁺ on mitochondrial Ca²⁺ cycling, which results in a decreased extramitochondrial Ca²⁺. Indeed, if this situation *in vitro* has relevance *in vivo*, one could imagine that alterations in cytosolic Mg²⁺ concentrations could be important in the regulation of mitochondrial Ca²⁺ cycling. Of particular relevance could be the observation that cytosolic free Mg²⁺ concentration decreases by 130 μ M after the addition of phenylephrine to hepatocytes (Corkey & Hale, 1982; Coll *et al.*, 1982).

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