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Manganese and calcium efflux kinetics in brain mitochondria

Relevance to manganese toxicity

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Manganese shares the uniport mechanism of mitochondrial calcium influx, accumulates in mitochondria and is cleared only very slowly from brain. Using dual-label isotope techniques, we have investigated both Mn^{2+} and Ca^{2+} mitochondrial efflux kinetics. We report that (1) there is no significant Na^+-dependent Mn^{2+} efflux from brain mitochondria; (2) Mn^{2+} inhibits both Na^+-dependent and Na^+-independent Ca^{2+} efflux in brain, in a mode that appears to be primarily competitive and with apparent K_i values of 5.1 and 7.9 nmol/mg respectively; and (3) Ca^{2+} does not appear to inhibit Mn^{2+} efflux from brain mitochondria. Findings (1) and (2) suggest the possibility of mitochondrial accumulation of both Mn^{2+} and Ca^{2+} in Mn^{2+}-intoxicated brain.

INTRODUCTION

Although it has long been known that Mn^{2+} is taken up by mitochondria over the Ca^{2+} uniporter (Chance, 1965; Lehninger, 1972), little attention has been paid to mitochondrial efflux, particularly in the brain. There are two reasons for undertaking such an investigation: first, the mechanisms of mitochondrial Ca^{2+} efflux are still somewhat controversial (Wingrove & Gunter, 1986; Crompton *et al.*, 1986), and an examination of Ca^{2+}–Mn^{2+} interactions over these mechanisms may help to characterize them more precisely, as it helped to characterize the uniporter (Vinogradov & Scarpa, 1973).

Secondly, Mn^{2+} toxicity remains a real concern (Roels *et al.*, 1987; Cawte *et al.*, 1987; Ferraz *et al.*, 1988). The brain is a major target tissue in manganism, with extremely slow Mn^{2+} clearance (Dastur *et al.*, 1971), and Mn^{2+} has been shown to accumulate *in vivo* in the mitochondria of brain areas associated with neurological symptoms of manganism (Liccione & Maines, 1988). Since Mn^{2+} uptake over the uniporter is slow (Vainio *et al.*, 1970; Puskin *et al.*, 1976; Gunter *et al.*, 1978), mitochondrial accumulation may reflect efflux kinetics that are even slower.

In addition, Mn^{2+} alters mitochondrial Ca^{2+} influx kinetics in brain and liver (Konji *et al.*, 1985); if the two ions share efflux mechanisms, elevated mitochondrial Mn^{2+} may be expected to alter Ca^{2+} efflux kinetics as well. Thus one consequence of elevated brain Mn^{2+} may be perturbation of normal intracellular Ca^{2+} distribution.

The two Ca^{2+} efflux mechanisms identified in mitochondria from a number of tissues are Na^+-dependent Ca^{2+} efflux, or Ca^{2+}/nNa^+ exchange (where $n = 2$ or 3), and Na^+-independent Ca^{2+} efflux, which exhibits the characteristics of a carrier or a gated pore (Wingrove & Gunter, 1986). In addition to these efflux mechanisms, mitochondrial suspensions *in vitro* may undergo permeability changes with concomitant loss (leakage) of ions (Beatrice *et al.*, 1982).

In this paper we report that Mn^{2+} inhibits Ca^{2+} efflux over both the Na^+-dependent and Na^+-independent mechanisms, and that Mn^{2+} does not share the Na^+-dependent Ca^{2+} efflux mechanism.

MATERIALS AND METHODS

Materials were those previously described (Gunter *et al.*, 1983; Wingrove & Gunter, 1986). Percoll was purchased from Pharmacia.

Rat brain mitochondria were prepared as follows. Female Sprague–Dawley rats (225–275 g) were killed by cervical dislocation followed by decapitation, and brains were removed within 20–30 s into ice-cold isolation medium (295 mM-mannitol, 65 mM-sucrose, 2 mM-Hepes, 1–2 mg of BSA/ml, 1 mM-EGTA, pH 7.4). After removing as much white matter as possible, the tissue was minced, then homogenized at 800 rev./min with 5 full strokes of a loose fitting Teflon pestle in a Thomas 'C' homogenizer, followed by 2–5 strokes with a pestle ground to a clearance of 0.2–0.3 mm. The crude mitochondrial pellets from four rats were resuspended in 18 ml of isolation medium, of which 0.6 ml aliquots were added to 10 ml of Percoll solution (250 mM-sucrose, 5 mM-Hepes, 0.1 mM-EGTA, 16–18% iso-osmotic Percoll, pH 7.5, at 0–4 °C) and centrifuged in a Beckman J-14 rotor for 45 min at 11000 *g* ($r_{av.}$ 8.15 cm). The loose mitochondrial pellet was removed with a wide-mouthed pipette and washed free of Percoll in isolation medium without EGTA in 50 ml tubes by centrifuging for 10 min at 13000 *g*, repeated two or three times. Respiratory control ratios (glutamate + malate) of the resulting mitochondria were approx. 8.0. The quality and purity of the preparation were further evaluated by electron microscopy.

Endogenous mitochondrial Ca^{2+} was measured spectrophotometrically with the metallochromic indicator Arsenazo III, after freezing and thawing followed by addition of the uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) (Wingrove & Gunter, 1986). Preparations typically contained 5–17 nmol of Ca^{2+}/mg of protein.

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Efflux rate measurements were made in one of two media. Medium A was 195 mM-mannitol, 65 mM-sucrose, 6–10 mM-potassium Hepes (pH 7.2), 10 mM-KCl, 0.05 mM-KH_2PO_4, 5 mM-potassium glutamate and 5 mM-potassium malate, pH 7.2, at room temperature. Medium B was 125 mM-KCl, 10 mM-potassium Hepes, 0.2 mM-ATP, 2.0 mM-$MgCl_2$, 0.05 mM-KH_2PO_4, 5 mM-potassium glutamate and 5 mM-potassium malate, pH 7.2.

Dual-label isotope studies

Mitochondria were suspended at 2–4 mg/ml in Medium A or B. After a 4 min equilibration period, $^{45}Ca^{2+}$ was added, followed 1 min later by $^{54}Mn^{2+}$ (when present). After 10 min the suspension was diluted to 0.7–1 mg/ml with medium containing EGTA (final concentration 2 mM) or EGTA plus Ruthenium Red (final concentration 0.004 mM). The final suspension contained 2 μCi of ^{45}Ca/ml and 1.6 μCi of ^{54}Mn/ml. In some preparations, the mitochondria were incubated at 0.74–1.0 mg/ml with no subsequent dilution. Following EGTA addition, aliquots were removed at 0.75–1 min intervals, and centrifuged for 30 s in a refrigerated Eppendorf tabletop centrifuge. Supernatants were poured off and counted for radioactivity in both a beta counter and a gamma counter, adjusting the resulting counts for the efficiencies of each counter for each ion.

Efflux rates were obtained by using least-squares-fit plots of intramitochondrial ion versus time after EGTA addition. The standard deviations (S.D.) of the slopes were used to weight the data points of Eadie–Hofstee and Dixon plots, using the weighting factors $(1/\text{S.D.})^2$ and $(1/[\text{S.D.} \cdot 1/v])^2$ respectively. Total intramitochondrial Mn^{2+} or Ca^{2+} was obtained by extrapolating initial efflux rates to the time of EGTA addition. This avoids the artificially high uptake values caused by external binding of ions to mitochondrial membranes. In Medium B, Ca^{2+} uptake was complete within 1 min (except for the smallest additions, which were 83% complete at 1 min), whereas Mn^{2+} uptake continued for up to 20 min.

For Na^+-dependent efflux, equal volumes taken from a single uptake sample were added to medium with or without Na^+ and were thereafter treated simultaneously. Final [Na^+] was kept below 10 mM in order to maintain linear efflux during the measurement period. Na^+-dependent efflux rates were obtained by subtracting the rate without Na^+ from the rate with Na^+. Standard deviations of Na-dependent efflux rates were obtained from the square root of the summed squared S.D. values of the rates with and without Na^+.

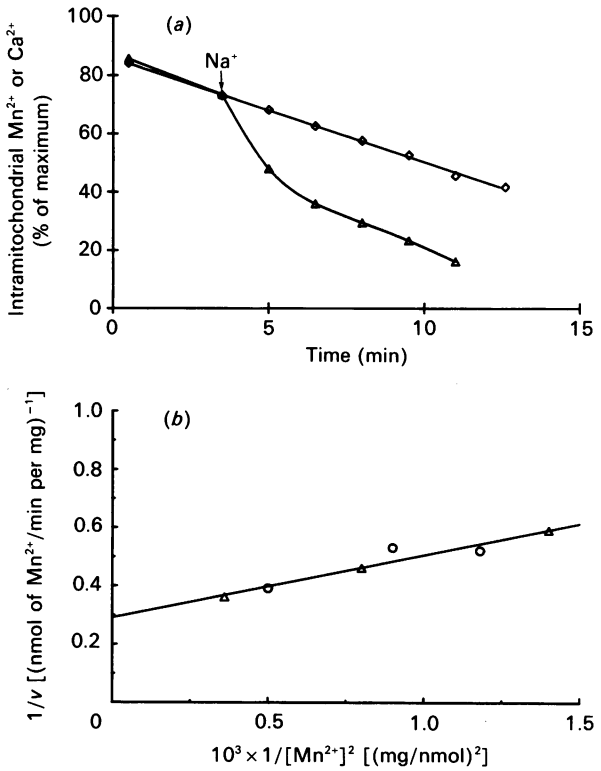

Fig. 1. Mn^{2+} does not exchange with Na^+ across the mitochondrial inner membrane

(*a*) Ca^{2+} and Mn^{2+} efflux in the presence of Na^+. Brain mitochondria were incubated at 4 mg/ml in mannitol/sucrose medium (Medium A). Ca^{2+} (△) and Mn^{2+} (◇) were added and efflux was initiated as described in the Materials and methods section. NaCl (0.01 ml) was added at the arrow (final [Na^+] 20 mM). Identical results were seen in KCl medium with and without 2 mM-Mg^{2+}. (*b*) Lineweaver–Burk (double-reciprocal) plot of Mn^{2+} efflux from brain mitochondria, incubated without added Ca^{2+}. After 10 min uptake time, equal portions of the solution were pipetted into two efflux tubes containing either medium (△) or medium plus 40 mM-NaCl (○), to a final protein concentration of 1.0 mg/ml and a final Na^+ concentration of 20 mM. All other procedures were as described in the Materials and methods section. The $V_{max.}$ in this preparation was 3.4, a typical value in the absence of added Mg^{2+} or ATP. The line is least-squares fitted to the data points.

RESULTS

Mn^{2+} efflux kinetics

The most important mitochondrial Ca^{2+} efflux mechanism in excitable tissues is Na^+-dependent efflux (Ca^{2+}/nNa^+ exchange), which in brain may be ten or more times faster than Na^+-independent efflux (Nicholls & Crompton, 1980). Fig. 1(*a*) shows clearly that the rate of Mn^{2+} efflux does not change after addition of Na^+ to a suspension of brain mitochondria, whereas $^{45}Ca^{2+}$ in the same samples responds strongly to Na^+ addition. Fig. 1(*b*) shows that Mn^{2+} efflux occurs at similar rates in medium with or without Na^+. We conclude that Mn^{2+} does not exchange with Na^+ across the inner membrane of brain mitochondria.

In the presence of physiological levels of Mg^{2+} (2 mM), Mn^{2+} efflux from brain mitochondria occurs with a $V_{max.}$ equal to the $V_{max.}$ for Ca^{2+} efflux of 1.0–2.0 nmol/mg (Fig. 2). (In the absence of Mg^{2+}, the Mn^{2+} $V_{max.}$ averages between 2.0 and 3.0 nmol/min per mg; see Fig. 1*b*.) The apparent K_m for Mn^{2+} in this preparation is 11.4 nmol/mg, nearly twice that for Ca^{2+}. Hill plots (results not shown) of Mn^{2+} efflux yielded Hill coefficients of 2.021 ± 0.049 (mean ± S.D. of four separate determinations), indicating second-order efflux.

Effects of Mn^{2+} on Ca^{2+} efflux

Figs. 3(*a*) and 3(*b*) show that Mn^{2+} inhibits Na^+-independent Ca^{2+} efflux in brain mitochondria. Na^+-independent Ca^{2+} efflux typically decreased by 40–60%, and was saturating at about 20 nmol of internal Mn^{2+}/mg of protein (Fig. 3*a*). In the Eadie–Hofstee plot shown in

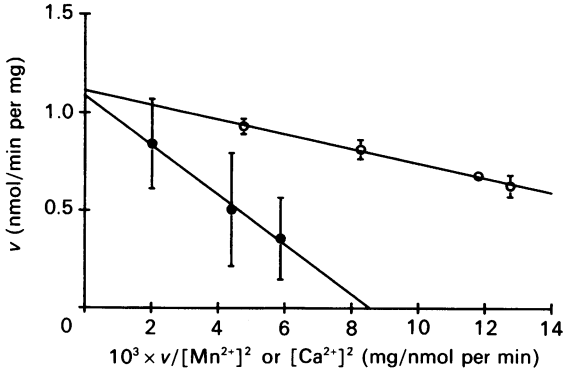

Fig. 2. Na^+-independent Mn^{2+} and Ca^{2+} efflux from brain mitochondria

Eadie–Hofstee plot of Na^+-independent Mn^{2+} (●) and Ca^{2+} (○) efflux from brain mitochondria. The Mn^{2+} preparation contains trace amounts of $^{45}Ca^{2+}$ (3.2 or 6.3 nmol/mg of protein); the Ca^{2+} preparation contains no added Mn^{2+}. Mitochondria were incubated at 0.74 mg/ml in Medium B and efflux was initiated with 2 mM-EGTA plus 4 μM-Ruthenium Red. The error bars reflect the S.D. values of efflux rates and are used to weight the data points as described in the Materials and methods section. In this preparation, $V_{max.}$ values were 1.09 nmol/min per mg for Mn^{2+} and 1.1 nmol/min per mg for Ca^{2+} and K_m values were respectively 11.4 and 6.0 nmol/mg. The lines are least-squares fitted to the data points.

Fig. 3(*b*), the convergence of the two Ca^{2+} plots near the ordinate indicates that the inhibition is primarily competitive. The K_i is calculated from the ratio of the slopes, using the second-order Eadie–Hofstee equation:

$$V = V_{max.} - K_m^2(V/[Ca^{2+}]^2)$$

and assuming simple competitive inhibition, where the $(K_m)^2_{app.} = K_m^2(1+[I]/K_i)$; therefore the ratio of the two slopes $= (K_i+[Mn^{2+}]_1)/(k_i+[Mn^{2+}]_2)$. The mean K_i from two independent experiments was 7.87 ± 0.68 nmol of Mn^{2+}/mg of protein.

Fig. 4 shows that Mn^{2+} inhibits Na^+-dependent Ca^{2+} efflux. As with inhibition of Na^+-independent efflux, the effect saturates at 15–25 nmol of internal Mn^{2+}/mg of protein. In the Dixon plot shown in Fig. 4, the intersection of the two Ca^{2+} plots above the abscissa suggests that the inhibition is primarily competitive (London & Shaw, 1974). Assuming competitive inhibition, the Dixon equation yields an apparent K_i in this preparation of 5.05 nmol of Mn^{2+}/mg of protein. Na^+-dependent Ca^{2+} efflux is also inhibited by Mn^{2+} in liver mitochondria (Gunter *et al.*, 1989).

In the experiments reported here, with physiological concentrations of Mg^{2+} in the media, Mn^{2+} added 1 min after Ca^{2+} enhanced total Ca^{2+} uptake into brain mitochondria by 6–15%, with a $K_{0.5}$ of 11.2 ± 3.4 μM-Mn^{2+} (results not shown).

Effects of Ca^{2+} on Mn^{2+} kinetics

In accordance with previous findings in liver mitochondria (Chance & Mela, 1966; Vainio *et al.*, 1970; Vinogradov & Scarpa, 1973), external Ca^{2+} stimulated Mn^{2+} uptake into brain mitochondria, measured after 10 min, by more than 30% (Fig. 5). This effect became

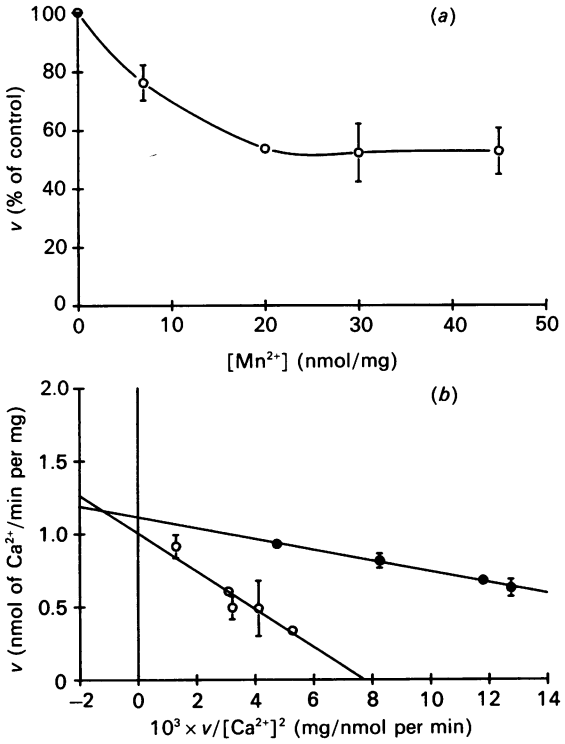

Fig. 3. Inhibition by Mn^{2+} of Na^+-independent mitochondrial Ca^{2+} efflux

(*a*) Efflux rates are expressed as percentages of rates at zero Mn^{2+}. Data points represent the means ± S.D. of at least three determinations. The $[Ca^{2+}]$ ranged from 12 to 47 nmol/mg of protein. (*b*) Eadie–Hofstee plot of Na^+-independent Ca^{2+} efflux in the presence (○) and absence (●) of Mn^{2+} (23 nmol/mg of protein). Conditions and error bars are as in Fig. 2. The lines are least-squares fitted to the weighted data.

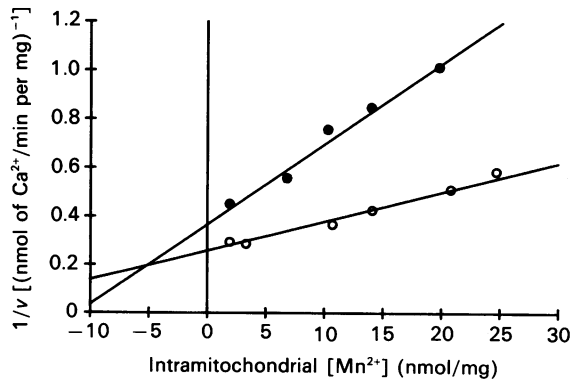

Fig. 4. Inhibition by Mn^{2+} of Na^+-dependent Ca^{2+} efflux: Dixon plot

Mitochondria were incubated in Medium B without Mg^{2+}, and ions were added as described in the Materials and methods section; equal portions of uptake samples were added to medium containing EGTA/Ruthenium Red with or without NaCl (final $[Na^+]$ 4 mM). Na^+-dependent efflux rates were obtained by subtracting the rates without Na^+ from those with Na^+ present. The least-squares line is fitted to the data points, which are weighted as described in Fig. 3(*b*) and in the Materials and methods section. $[Ca^{2+}]$: ●, 27.3 nmol/mg of protein; ○, 37.3 nmol/mg.

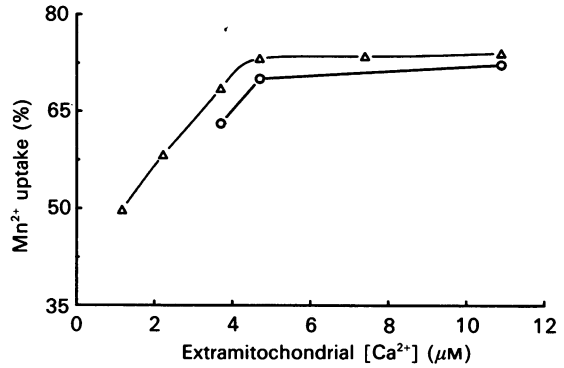

Fig. 5. Ca^{2+} enhancement of Mn^{2+} uptake

Mitochondria were incubated in Medium B at 0.7–1.0 mg/ml and Mn^{2+} was added 1 min after Ca^{2+}; all other procedures were as described in the Materials and methods section. Extramitochondrial [Ca^{2+}] at the time of Mn^{2+} addition was estimated based on the percentage uptake of labelled Ca^{2+} in the absence of added Mn^{2+}. [Mn^{2+}]: △, 40 nmol/mg of protein; ○, 10 nmol/mg.

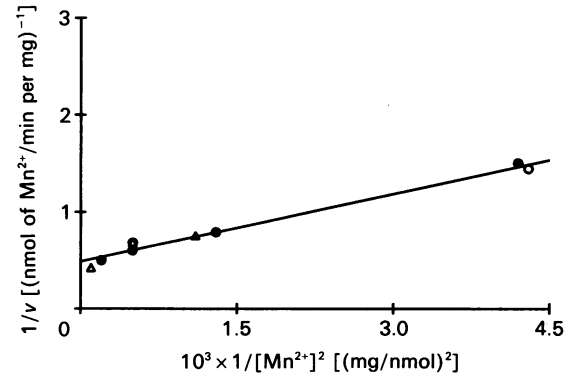

Fig. 6. Na^+-independent Mn^{2+} efflux at three concentrations of Ca^{2+}: Lineweaver–Burk plot

Mitochondria were incubated at 0.55 mg/ml in KCl medium (medium B), with 0.5 mM-Mg^{2+}, and efflux was initiated with 2 mM-EGTA. The same results were observed in the absence of Mg^{2+} and, with considerably more scatter, at 2 mM-Mg^{2+}. The line is least-squares fitted to the data points. [Ca^{2+}]: ●, 13.3 nmol/mg of protein; ○, 20 nmol/mg; △, 73.7 nmol/mg.

saturated at 4–5 μM extramitochondrial Ca^{2+}, with a K_a of 2.2 μM.

Although Mn^{2+} strongly inhibits Ca^{2+} efflux from both brain and liver mitochondria, and although Ca^{2+} inhibits Na^+-independent Mn^{2+} efflux from liver mitochondria (results not shown), internal Ca^{2+} concentrations of up to 70 nmol/mg do not significantly affect Na^+-independent Mn^{2+} efflux from brain mitochondria (Fig. 6).

DISCUSSION

Kinetic basis for Mn^{2+} accumulation in brain mitochondria

Mn^{2+} is normally admitted to the brain in very small quantities; rigorously controlled at the levels of absorption and excretion, its half-life in blood is of the order of a few minutes (Oberdoerster & Cherian, 1985), as it is taken up by the liver and excreted into bile. With continuing exposure, however, particularly via inhalation (thus bypassing homeostatic control mechanisms), brain Mn becomes elevated, accumulating preferentially in the basal ganglia (Suzuki *et al.*, 1975; Fahn, 1976; Autissier *et al.*, 1982; Newland *et al.*, 1988). Brain Mn clearance may take up to a year (Dastur *et al.*, 1971).

After experimental exposure *in vivo*, Mn^{2+} has been found to be taken up into mitochondria of liver (Maynard & Cotzias, 1954) and brain (Liccione & Maines, 1988). These authors observed higher levels of Mn in striatal mitochondria than in those isolated from cortex. We have shown that this accumulation may be related to a combination of Ca^{2+}-enhanced Mn^{2+} influx and extremely slow Mn^{2+} efflux from brain mitochondria.

The marked enhancement by Ca^{2+} of Mn^{2+} uptake *in vitro* was observed in liver by Chance & Mela (1966), Vainio *et al.* (1970) and Vinogradov & Scarpa (1973), and reported in brain by Konji *et al.* (1985). However, the lowest Ca^{2+} concentration employed in any of these studies was 23 μM, well above physiological levels. We have found that much lower concentrations of extramitochondrial Ca^{2+} stimulate the uptake of Mn^{2+} by over 30%. Although it is generally agreed that most minute-to-minute Ca^{2+} buffering in the cell is carried out at sub-micromolar basal Ca^{2+} concentrations by endoplasmic reticulum (Becker *et al.*, 1980), local 'spikes' of cytosolic Ca^{2+} (Cobbold & Rink, 1987) may reach micromolar levels, capable of triggering mitochondrial influx of both Ca^{2+} and Mn^{2+} (Unitt *et al.*, 1989).

It follows that excitable tissue, experiencing frequent Ca^{2+} spikes, is likely to accumulate mitochondrial Mn. Moreover, the striatum, which preferentially accumulates Mn, is among the most synaptically active areas of brain (Kandel & Schwartz, 1985).

Although such Ca^{2+} spikes are short-lived, their influence on Mn^{2+} transport is prolonged because of the nature of the mitochondrial uniporter that transports cations into mitochondria. Considerable evidence has accumulated to support the existence of separate sites on the uniporter for transport and activation (Vinogradov & Scarpa, 1973). Once bound to the activation site, Ca^{2+} remains bound for the order of minutes (Kroner, 1988); hence activation of transport of Mn^{2+} as well as of Ca^{2+} continues even after a Ca^{2+} spike has passed.

The extremely slow kinetics of Mn^{2+} efflux from brain mitochondria, characterized here for the first time, may be expected to contribute both to Mn accumulation in brain mitochondria and to its slow clearance from brain tissue. Most important is the lack of any Na^+-dependent Mn^{2+} efflux, despite the report of Konji *et al.* (1985) to the contrary. This conclusion is supported by the results of eight independent experiments in various media. Brain mitochondria thus possess a mechanism for Mn^{2+} influx, but no mechanism for Mn^{2+} clearance other than the slow Na^+-independent mechanism. At this time it is not known whether elevated mitochondrial Mn, in itself, is injurious or without effect, or even protective. However, many researchers have speculated that Mn^{2+} oxidation to Mn^{3+} by oxygen free radicals is the initiating event in manganese toxicity (Halliwell, 1984; Graham, 1984; Archibald & Tyree, 1987; Donaldson, 1987), and the mitochondrial electron transport system is one of the few known significant sources of superoxide within the cell (Forman & Boveris, 1982). Further, Mn^{2+} has been found to bind after uptake to the inner mitochondrial

membrane (Gunter *et al.*, 1975), where the electron transport system is located.

Mn^{2+} enhancement of Ca^{2+} influx

Mn^{2+} stimulated Ca^{2+} uptake into brain mitochondria by up to 15% in the presence of 2.0 mM-Mg^{2+}. Our results in brain thus support previous results in liver (Hughes & Exton, 1983; Allshire *et al.*, 1985), suggesting that the uniporter influx mechanism is similar in the two tissues. In addition, this finding is consistent with the two-site uniporter model of Vinogradov & Scarpa (1973) discussed above. The observed phenomena suggest that (1) Mn^{2+}, Ca^{2+} and Mg^{2+} all bind to the activation site; (2) Mn^{2+} and Ca^{2+} bind more strongly to the activation site than does Mg^{2+}; (3) Mg^{2+} occupation of the site inhibits Ca^{2+} uptake; and (4) Mn^{2+} inhibits Ca^{2+} uptake, but less than does Mg^{2+}. Thus, added micromolar Mn^{2+} displaces millimolar Mg^{2+} from activation sites, partially disinhibiting Ca^{2+} influx. It is possible that the observed enhancement of Ca^{2+} uptake is nothing more than Mn^{2+} inhibition of Ca^{2+} efflux. However, in the absence of Mg^{2+}, Mn^{2+} inhibition of Ca^{2+} efflux remains strong and Ca^{2+} uptake is inhibited (Allshire *et al.*, 1985). Further investigation of Mg^{2+}–Mn^{2+}–Ca^{2+} interactions over all three transport mechanisms will be necessary in order to resolve this question fully.

Mn^{2+} inhibition of Ca^{2+} efflux

The competitive nature of Mn^{2+} inhibition of Ca^{2+} efflux over the Na^+-independent mechanism is noteworthy for two reasons: first, the K_i (8 nmol/mg) is low enough to make the inhibition physiologically relevant, at least potentially; second, the phenomenon of competition for binding sites is strong evidence that Na^+-independent efflux is a true carrier or gated-pore-mediated efflux mechanism and not simply ion leakage. Mn^{2+} inhibits Na^+-independent Ca^{2+} efflux in liver mitochondria as well as in brain, also in a primarily competitive mode but with a larger K_i, indicating stronger inhibition in brain (Gunter *et al.*, 1989).

Mn^{2+} inhibition of Na^+-dependent Ca^{2+} efflux is even stronger than that of the Na^+-independent mechanism (K_i 5 nmol/mg). The competition between Mn^{2+} and Ca^{2+} for transport sites on the matrix face of the Ca^{2+}/nNa^+ exchanger suggests that further investigation (for example, of competition for Ca^{2+} binding sites on the external face of the membrane) may yield more information on the nature of these sites (see Hayat & Crompton, 1982).

From a toxicological standpoint, Mn^{2+} inhibition of Na^+-dependent Ca^{2+} efflux raises the possibility that elevated brain Mn^{2+} may increase the Ca^{2+} content of brain mitochondria. In some pathological states that lead to excess cytosolic Ca^{2+}, mitochondria are known to take up large quantities of Ca^{2+}. The combination of Mn^{2+} enhancement of Ca^{2+} influx, together with inhibition of Ca^{2+} efflux over both efflux mechanisms, may increase mitochondrial Ca^{2+} enough to create significant oxidative stress (Olafsdottir *et al.*, 1988). These authors found that uptake of large quantities of Ca^{2+} by mitochondria of cultured hepatocytes was accompanied by a decrease in mitochondrial GSH, which in turn was a better predictor of cell death than increased cytosolic Ca^{2+}. Further studies, both *in vivo* and *in vitro*, will be necessary before it can be determined whether the potential for Ca^{2+} accumulation suggested by Mn^{2+}/Ca^{2+} kinetics may actually be realized in cases of Mn toxicity.

It is curious that the role of the mitochondrion in Mn toxicity has received so little attention. We have shown a kinetic basis for the observed accumulation of Mn in mitochondria, together with a means by which elevated brain Mn^{2+} may perturb intracellular Ca^{2+} distribution. There thus appear to be sound reasons for looking more closely at both direct and indirect effects of Mn on brain mitochondrial function.

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