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# Manganese stimulates calcium flux through the mitochondrial uniporter

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**$Mn^{2+}$ alters the balance between the simultaneous uptake and release of $Ca^{2+}$ across the mitochondrial inner membrane toward a lower external level. Addition of as little as 0.5 μM $Mn^{2+}$ to energised mitochondria from rat liver, rat heart or guinea-pig brain changed the level at which they buffered $Ca^{2+}$ in the medium. That extramitochondrial $Mn^{2+}$ was responsible was suggested by a partial decay in the shift in $Ca^{2+}$ steady state at a rate similar to the rate at which $Mn^{2+}$ was accumulated by the mitochondria. The alteration of transmembrane $Ca^{2+}$ distribution by $Mn^{2+}$ required that both $Mg^{2+}$ and $P_i$ be present, and was almost maximal at $Mg^{2+}$ and $P_i$ levels in the physiological range. Substitution of spermine or $Ni^{2+}$ for $Mg^{2+}$, or acetate for $P_i$, abolished the effect. In contrast to $Sr^{2+}$, $Mn^{2+}$ did not inhibit either EGTA- or Ruthenium red-induced release of $Ca^{2+}$ from the mitochondria. However, when flux through the uniporter was rate-limiting, $Mn^{2+}$ accelerated $Ca^{2+}$ uptake. The stimulation showed hyperbolic kinetics, with an element of competition discernible in the $Mn^{2+}$-$Mg^{2+}$ interaction. Thus, extramitochondrial $Mn^{2+}$ at levels occurring in vivo can alter the mitochondrial 'set-point' by stimulating $Ca^{2+}$ influx through the uniporter.**

## Introduction

The distribution of $Ca^{2+}$ resulting from simultaneous movement of the cation in both directions across the mitochondrial inner membrane is a kinetic equilibrium responsive to change in influx or efflux [1]. Energised mitochondria normally maintain free $Ca^{2+}$ at 1 μM or less in vitro. Following additions of either $Ca^{2+}$ or chelator, a compensatory net uptake or release of $Ca^{2+}$ enables the mitochondria to restore the p*Ca* of the medium to this 'set-point' [2].

In the absence of effects on efflux, the inhibition of $Ca^{2+}$ uptake on the uniporter by $Sr^{2+}$ [3] or $Mn^{2+}$ [4] would be predicted to alter the mitochondrial set-point to a lower external p*Ca* on this basis. However, as described here and elsewhere [5,6], micromolar amounts of $Sr^{2+}$ and $Mn^{2+}$ shift the level at which mitochondria buffer $Ca^{2+}$ to higher rather than lower p*Ca* values. The effect of $Sr^{2+}$ has been traced to its potent inhibition of $Ca^{2+}$ efflux [5]. Since $Sr^{2+}$ accumulated by the mitochondria is not released again at any significant rate, the merely temporary inhibition of $Ca^{2+}$ influx by $Sr^{2+}$ will not contribute to the new steady state. In contrast, the efflux of $Ca^{2+}$ revealed on adding Ruthenium red to inhibit the uniporter is unaffected by $Mn^{2+}$, while the ability of $Mn^{2+}$ to change the mitochondrial set-point was sensitive to inhibition of the uniporter [6].

In this study, we show directly that in the presence of $Mg^{2+}$ and $P_i$ $Mn^{2+}$ stimulates $Ca^{2+}$

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Abbreviations: HEDTA, *N*-hydroxyethylethylenediaminetriacetate, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; p*Ca*, $-\log[Ca^{2+}]$

flux through the uniporter of liver, heart and brain mitochondria, and have analysed the kinetic properties of the phenomenon.

## Materials and Methods

*Materials.* $^{45}CaCl_2$, $^{89}SrCl_2$ and ACS scintillation counting fluid were from Amersham; dinonylphthalate and di-*n*-butylphthalate were products of BDH. Bovine serum albumin was a fraction V preparation from Sigma. Ruthenium red from Sigma was purified [7] and arsenazo III (Sigma) recrystallised [8] before use. Other reagents were of the highest quality commercially available.

*Mitochondria.* Male Wistar rats weighing 150–200 g were stunned and the livers or hearts immediately removed. Liver mitochondria were isolated by differential centrifugation after homogenisation of the minced tissue in 210 mM mannitol/70 mM sucrose/1 mM EGTA/10 mM Hepes-Tris (pH 7.3)/0.5% bovine serum albumin. Hearts were homogenised by treatment for 15 s with an Ultra-Turrax TP 18-10 tissue processor in a medium in which 120 mM KCl replaced mannitol/sucrose as the main osmotic support. Guinea-pig brain mitochondria were prepared from cerebral cortex of 4–8-week-old animals in the mannitol/sucrose medium. All mitochondria were washed three times in media lacking EGTA, then resuspended to 60 mg protein · $ml^{-1}$ (liver), 40 mg · $ml^{-1}$ (heart) or 20 mg · $ml^{-1}$ (brain).

Mitoplasts were prepared by treatment of freshly isolated rat liver mitochondria with digitonin [9] at a rate of 1 mg/8.5 mg mitochondrial protein, then washed twice in detergent-free medium to remove disrupted outer membranes and intermembrane material. All steps of the isolation procedures were carried out at 2–4°C. Protein was determined by a biuret method [10] using bovine serum albumin as a standard.

*pCa measurement.* Free $Ca^{2+}$ was measured in a water-jacketed glass reaction vessel using a $Ca^{2+}$-selective electrode (Phillips IS 561-Ca) and a KCl reference electrode (Beckman). The vessel contents were stirred rapidly and smoothly with a magnetic stirrer and circular follower (diameter 0.8 cm). The output from the electrode was relayed from a pH/voltmeter (IM-555; Instrumentarium, Helsinki) to a strip-chart recorder. A voltage-bucking device provided additional offset.

Electrode response was calibrated over the p*Ca* range 5.0–6.4 in the presence of 0–1.5 mM free $Mg^{2+}$ with a series of $Ca^{2+}$ buffers based on HEDTA (50 mM HEDTA, 50 mM KCl, 10 mM Mops-Tris, $MgCl_2$ and $CaCl_2$ as required, and adjusted to pH 7.0 at 30°C). Calculations of free divalent cation assumed $K'$ values of $2.57 \cdot 10^5$ for Ca-HEDTA and $1.12 \cdot 10^3$ for Mg-HEDTA at pH 7.0 and 30°C [2]. The potential difference-p*Ca* relationship measured was extremely stable during series of experiments and from day to day, and in contrast to the difficulties reported by Hughes and Exton [6], the electrode showed a low sensitivity to $Mn^{2+}$ over the concentration range used.

Mitochondrial $Ca^{2+}$ transport was measured at 30°C in a final volume of 2.1 ml. The basic medium comprised 150 mM sucrose/40 mM choline chloride/0.1% bovine serum albumin/10 mM Mops-Tris (pH 7.0) (30°C), to which was added 1 mg mitochondrial protein · $ml^{-1}$ in the reaction vessel. Other additions from concentrated stocks were as noted in the legends.

As well as repeated flushing with distilled water between experiments, surfaces of the reaction vessel and electrodes were washed with mitochondria after use of Ruthenium red, and with 40% ethanol after use of reagents only sparingly soluble in water.

*$^{45}Ca$ and $^{89}Sr$ fluxes.* The standard incubation medium used contained 10 mM Mops-Tris (pH 7.0), 0.1% bovine serum albumin, 0.2 $\mu$Ci $^{45}Ca$ or $^{89}Sr$ · $ml^{-1}$ and either 150 mM sucrose and 40 mM choline chloride or 120 mM KCl, together with other additions indicated in the figure legends. Uptake of divalent cation was measured at 30°C. After mitochondria were added (1 mg · $ml^{-1}$) to begin the reaction, samples were removed and either filtered or centrifuged at appropriate times. 100-$\mu$l aliquots of the reaction mixture were filtered through Millipore filters (0.45 $\mu$m pore size) and the filters were washed with 1 ml ice-cold label-free medium. Alternatively, 300 $\mu$l of the incubate were added to 400 $\mu$l di-*n*-butylphthalate/dinonylphthalate (2 : 1, v/v) and centrifuged for 1 min in a bench centrifuge (model 5414, Eppendorf). Radioactivity on the filters or in 100-$\mu$l samples of the aqueous upper phase was then counted in 5 ml of a scintillation cocktail (ACS, Amersham) using

a scintillation spectrophotometer (LKB-Wallac, Turku).

*$Mn^{2+}$ fluxes.* The uptake of $Mn^{2+}$ by rat liver mitochondria was followed at 30°C in a medium comprising 200 mM sucrose/20 mM KCl/1 mM $P_i$-Tris/1.5 mM $MgCl_2$/2 mM succinate-Tris/10 mM Hepes-Tris (pH 7.0)/1 μM rotenone/1 μg oligomycin · $ml^{-1}$/20 μM arsenazo III/1 mg mitochondrial protein · $ml^{-1}$. After the mitochondria had reduced $Ca^{2+}$ in the medium to 1 μM or less, 10–50 μM $MnCl_2$ was added and absorbance changes were measured with an Aminco DW-2 dual wavelength spectrophotometer using the wavelength pair 641-666 nm. Under these conditions, the level of free $Mg^{2+}$ in the medium was approximately constant and the arsenazo III predominantly reported free $Mn^{2+}$.

## Results

*$Mn^{2+}$ shifts the mitochondrial set-point to higher pCa values*

Fig. 1 shows the effect of several divalent cations on the level at which energised rat liver mitochondria maintained free $Ca^{2+}$ in a $Mg^{2+}$-containing medium. After additions of $Ca^{2+}$ the p*Ca* was quickly restored to its original value (trace a). $Ba^{2+}$ caused a small electrode response (trace b) and was slowly accumulated without measurably affecting the distribution of $Ca^{2+}$. $Sr^{2+}$ elicited a large electrode response (trace c), after which uptake of the cation could be followed until a new steady state was established at a higher p*Ca* than before. Addition of $Mn^{2+}$ (trace d) under these conditions caused a rapid and large change in the mitochondrial set-point which was complete within about 30 s and then partly decayed. The half-time of decay corresponded well with that of $Mn^{2+}$ uptake by the mitochondria as measured in parallel experiments using arsenazo III (not shown), suggesting that the effect on set-point requires extramitochondrial rather than matrix $Mn^{2+}$. That the decay was only partial and soon completed implies that $Mn^{2+}$ exerted control over the $Ca^{2+}$ distribution by binding at sites with an affinity greater than that of the transport sites. A reproducible shift in set-point was measurable at $Mn^{2+}$ concentrations as low as 0.5 μM, and was maximal at 30 μM with an apparent $K_m$ of about 8 μM at 30°C in a medium which contained 1.5 mM $Mg^{2+}$ and 1 mM $P_i$.

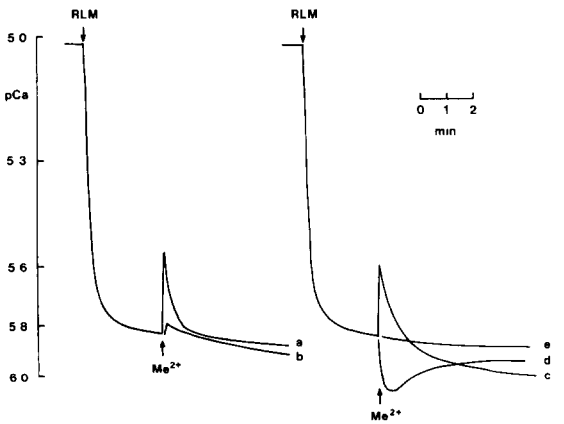


Fig 1 Effects of divalent cations on the set-point of rat liver mitochondria Free $Ca^{2+}$ was measured at 30°C in the basic medium (described under Materials and Methods) supplemented with 1 mM $P_i$-Tris, 1 5 mM $MgCl_2$, 1 mM succinate-Tris, 1 μM rotenone and 1 μg oligomycin · $ml^{-1}$ Additions. rat liver mitochondria (RLM), 1 mg · $ml^{-1}$, divalent cations ($Me^{2+}$), either (a) 5 μM $CaCl_2$, (b) 10 μM $BaCl_2$ (c) 10 μM $SrCl_2$ or (d) 10 μM $MnCl_2$. Trace e, no cation added

In experiments similar to that described in Fig. 1 but where $Mg^{2+}$ had been omitted from the medium, the effect of $Sr^{2+}$ on mitochondrial set-point was reduced while that of $Mn^{2+}$ was almost abolished. Moreover, the action of $Mn^{2+}$ on set-point could not be restored by adding either 0.3 mM spermine or 10 μM $Ni^{2+}$ (results not shown).

Responses to $Mn^{2+}$ of the p*Ca* maintained by respiring mitoplasts were similar to those of the parent liver mitochondria (not shown), so the concentration dependence measured reflects that of the mechanism in the inner membrane and precludes an involvement of intermembrane components. Qualitatively similar effects of $Mn^{2+}$ on mitochondria from rat heart and guinea-pig brain were found.

Movements of $^{45}Ca$ following an addition of $Mn^{2+}$ to respiring rat liver mitochondria (Fig. 2) resembled those observed using the electrode. In the presence of 5 and 15 μM $Sr^{2+}$, the redistribution, though somewhat reduced, could still be seen. Therefore, since $Mn^{2+}$ and $Sr^{2+}$ did not seem to compete strongly in exerting their effects on $Ca^{2+}$ transport and since $Sr^{2+}$ inhibits $Ca^{2+}$ efflux, it

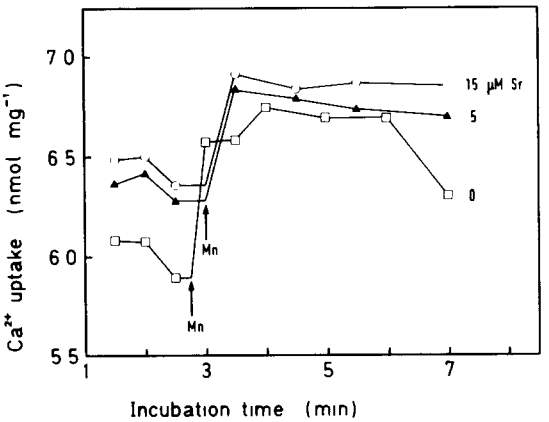


Fig. 2 $Mn^{2+}$-induced redistribution of $Ca^{2+}$ across the mitochondrial membrane in the presence of $Sr^{2+}$. Uptake of $^{45}Ca$-labelled endogenous $Ca^{2+}$ was measured at 30°C in a KCl-based medium (see Materials and Methods) which included 1 mM $P_i$-Tris, 1.5 mM $MgCl_2$, 2 mM succinate-Tris, 1 μM rotenone and 1 μg oligomycin·$ml^{-1}$, and to which rat liver mitochondria (0.75 mg·$ml^{-1}$) were added $SrCl_2$ was either absent (□) or present at 5 μM (▲) or 15 μM (○), and 10 μM $MnCl_2$ was added as indicated. Mitochondria were separated from samples of the incubate by centrifugation.

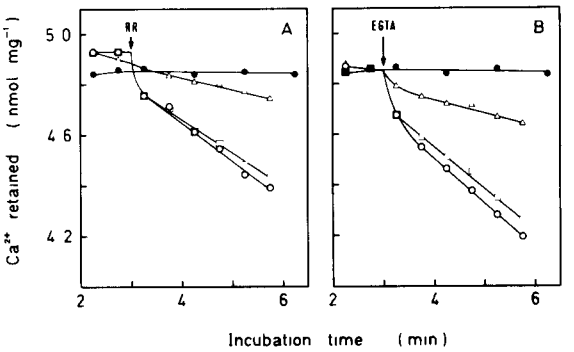


Fig. 3. Effects of $Sr^{2+}$ and $Mn^{2+}$ on $Ca^{2+}$ efflux. Rat liver mitochondria (1.9 mg·$ml^{-1}$) were incubated as in Fig. 2, except that endogenous $Ca^{2+}$ was supplemented with 5 μM $CaCl_2$. Net efflux from the mitochondria was begun by adding Ruthenium red (A· RR, 1 nmol·$mg^{-1}$) or EGTA (B· 1 mM) under control conditions (○) or in the presence of 40 μM $MnCl_2$ (□) or 40 μM $SrCl_2$ (△). The mitochondria retained $Ca^{2+}$ in the absence of Ruthenium red or EGTA (●)

appeared likely that $Mn^{2+}$ affected the transmembrane $Ca^{2+}$ distribution in some other way.

*Lack of effect of $Mn^{2+}$ on $Ca^{2+}$ efflux from energised mitochondria*

Direct measurement of $Ca^{2+}$ effluxes from rat liver mitochondria after addition of Ruthenium red (1 nmol·$mg^{-1}$) to inhibit influx on the uniporter or EGTA (1 mM) to chelate extramitochondrial $Ca^{2+}$ showed that both were inhibited by about 70% in the presence of 40 μM $Sr^{2+}$ (Fig. 3). $Mn^{2+}$ at 40 μM had very much smaller effects. Yet, since as little as 0.5 μM $Mn^{2+}$ measurably altered the set-point of the mitochondria under conditions as in Fig. 1, it was evident that the mechanism must indeed be other than through an inhibition of efflux. It was clear too that under these conditions $Mn^{2+}$ did not change the activity of $Ca^{2+}$ in the mitochondrial matrix as this would have been reflected in altered effluxes. Thus, in view of the lack of effect of $Mn^{2+}$ on efflux, the alteration of set-point implies that an acceleration of $Ca^{2+}$ influx was taking place instead.

*$Mn^{2+}$ effects on $Ca^{2+}$ influx*

In preliminary experiments to examine $Ca^{2+}$ influx under rate-limiting conditions we measured the rates at which rat heart mitochondria accumulated $Ca^{2+}$ from a medium with $Mg^{2+}$ (Fig. 4).

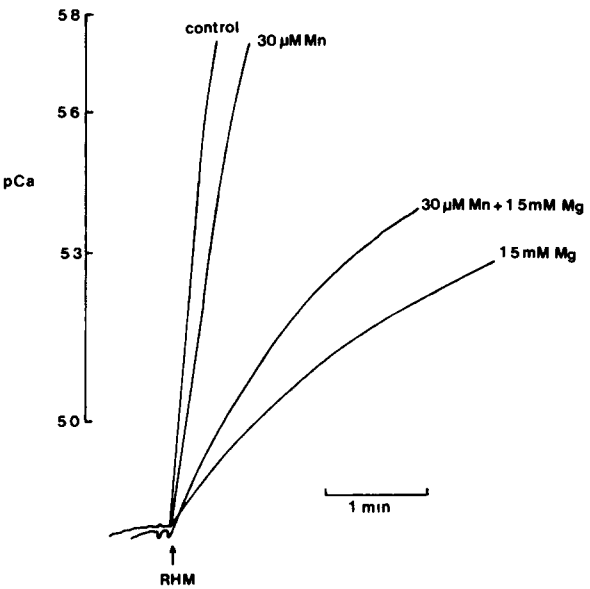


Fig. 4. Stimulation of $Ca^{2+}$ influx by $Mn^{2+}$. Uptake of $Ca^{2+}$ by rat heart mitochondria (RHM, 1.6 mg·$ml^{-1}$) was measured with $MgCl_2$ and $MnCl_2$ present as indicated. Other conditions were as in Fig. 1

The slow influxes of $Ca^{2+}$ under such conditions relative to the maximum capacity of respiration to reenergise the membrane ensured that effects of $Mn^{2+}$ on the uniporter could be observed. Fig. 4 shows that while 30 $\mu$M Mn inhibited $Ca^{2+}$ influx in the absence of $Mg^{2+}$, presumably through competition for transport sites [4], uptake of $Ca^{2+}$ from a medium containing 1.5 mM $Mg^{2+}$ was markedly enhanced by $Mn^{2+}$.

To measure $Ca^{2+}$ fluxes into rat liver mitochondria, uptake was begun by adding an excess of valinomycin to generate a $K^+$-diffusion potential across the inner membrane. In this way, rates measured would reflect fluxes through the uniporter under true rate-limiting conditions [11]. Incubation of mitochondria with 1.5 mM $Mg^{2+}$ and 10 $\mu$M $Mn^{2+}$ caused a much faster $Ca^{2+}$ influx than that with $Mg^{2+}$ alone (Fig. 5), while uptake of $Ca^{2+}$ from a medium lacking $Mg^{2+}$ was

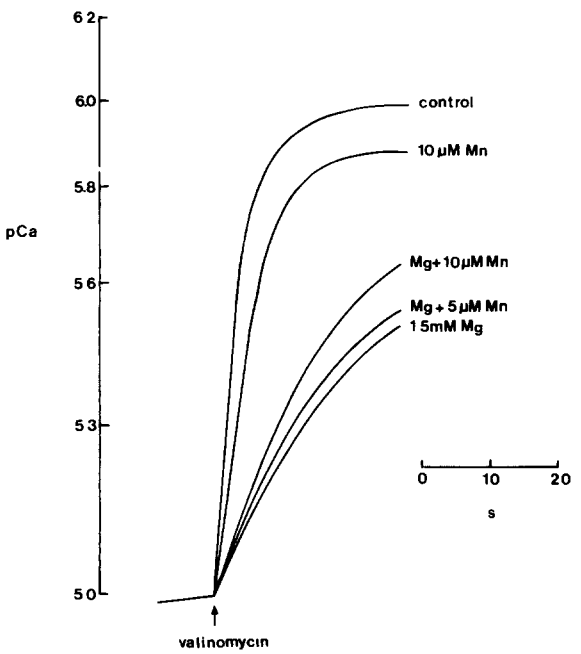


Fig. 5. $Mn^{2+}$ stimulation of $Ca^{2+}$ uptake by rat liver mitochondria. Mitochondria were incubated with rotenone (1 nmol·mg$^{-1}$) and antimycin A (0 1 $\mu$g·mg$^{-1}$) at 2°C for 5 min, then added to medium equilibrated at 30°C to a final concentration of 1 mg·ml$^{-1}$ The medium (see Materials and Methods) also contained 1 mM $P_i$-Tris, 1 $\mu$M rotenone, 1 $\mu$g oligomycin·ml$^{-1}$ and divalent cation as shown. Uptake was begun by adding excess valinomycin (4 $\mu$M)

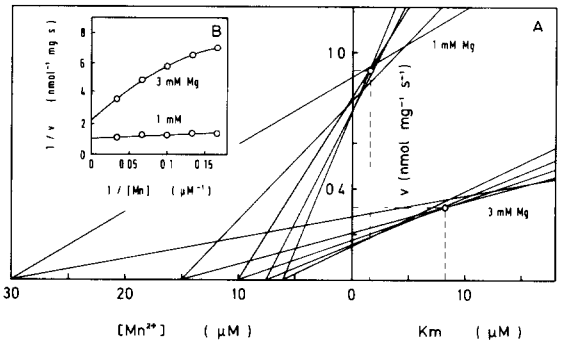


Fig. 6 Direct (A) and double-reciprocal (B) plots of $Ca^{2+}$ influx as a function of $Mn^{2+}$ concentration Rat liver mitochondria were added to medium containing $MnCl_2$ and 1 or 3 mM $MgCl_2$, followed 1 min later by valinomycin. Otherwise, conditions were as in Fig. 5

inhibited by $Mn^{2+}$. In these experiments, a distinct stimulation of $Ca^{2+}$ influx was measurable at $Mn^{2+}$ concentrations of 4 $\mu$M or greater. The apparent kinetic parameters were sensitive to $Mg^{2+}$ as reflected both in measurements of influx, $v$ (Fig. 6A), and in measurements of set-point (Fig. 7), revealing a mixed $Mn^{2+}$-$Mg^{2+}$ interaction. Furthermore, the kinetics of $Mn^{2+}$-stimulation of $v$ became increasingly hyperbolic as the $Mg^{2+}$ concentration was increased (Fig. 6B).

Since $v$ could not be measured at low external

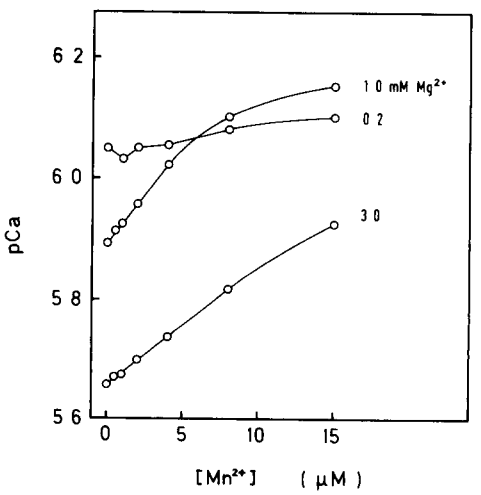


Fig 7. $Mg^{2+}$ requirement of the $Mn^{2+}$ effect on mitochondrial set-point The response of the set-point of rat liver mitochondria to $MnCl_2$ was measured at 30°C in the medium described in Fig. 1, but at various $MgCl_2$ concentrations

free $Ca^{2+}$ concentrations because the $Ca^{2+}$ buffers needed would also bind $Mn^{2+}$, a crude estimate of the kinetic parameters of the $Mn^{2+}$ effect at sub-micromolar $Ca^{2+}$ was attempted by extrapolation from data obtained over the range 5–15 $\mu$M $Ca^{2+}$. The apparent $K_m$ of $Mn^{2+}$ activation of influx increased with decreasing external $Ca^{2+}$ (Table I), and suggests a $K_m$ of 7–8 $\mu$M at 1 $\mu$M $Ca^{2+}$ – consistent with measurements of $Mn^{2+}$ effects on set-point (where the external $Ca^{2+}$ was usually about 1 $\mu$M), and close enough to the 0.2–1 $\mu$M free $Mn^{2+}$ measured in hepatocytes [12] to make the $Mn^{2+}$ effect potentially significant in the intact cell.

*Mechanism of $Mn^{2+}$ stimulation of the uniporter*

That the apparent $K_m$ of the $Mn^{2+}$ effect on mitochondrial $Ca^{2+}$ set-point agreed well with that estimated from measurements of $v$ where influx was rate-limiting provides further indirect evidence that the mechanism of the alteration of $Ca^{2+}$ distribution in the absence of ionophore and respiratory inhibitor other than rotenone was through a stimulation of the uniporter. Replacement of $Mg^{2+}$ by 0.3 mM spermine, which alters $Ca^{2+}$ uptake apparently through effects on the surface charge of the mitochondrial membrane [13], abolished the $Mn^{2+}$ effect on set-point. Neither could effects of $Mn^{2+}$ on set-point be distinguished when $Mg^{2+}$ was replaced by 10 $\mu$M $Ni^{2+}$. Likewise, the alteration in $Ca^{2+}$ distribution was no longer seen if $Mn^{2+}$ were replaced by 10 $\mu$M $Co^{2+}$. Instead, a slow release of accumulated $Ca^{2+}$ took place (not shown).

The effect of $Mn^{2+}$ on $Ca^{2+}$ distribution was markedly reduced if $P_i$ were omitted from the medium [6] or if its uptake by the mitochondria were inhibited with mersalyl (present study, results not shown). Addition of acetate did not restore the sensitivity of set-point to $Mn^{2+}$, implying that a low matrix $Ca^{2+}$ activity is required.

TABLE I

Effect of extramitochondrial free $Ca^{2+}$ on $Mn^{2+}$ stimulation of the uniporter

The rate of $Ca^{2+}$ uptake by rat liver mitochondria was measured as in Fig. 5 Apparent kinetic parameters were derived from direct plots of the data.

| $[Ca^{2+}]$ ($\mu$M) | $K'_m$ ($\mu$M) | $V_{max}$ (nmol·mg$^{-1}$·s$^{-1}$) |
|---|---|---|
| 6.03 | 5 98 | 0 881 |
| 8.62 | 4.43 | 1.15 |
| 10.3 | 1.66 | 1.19 |

## Discussion

*$Mn^{2+}$ stimulation of the uniporter*

Micromolar $Mn^{2+}$ was found to alter the set-point of isolated mitochondria to higher external p*Ca* values by stimulating $Ca^{2+}$ uptake on the uniporter rather than by inhibiting release. The kinetics of the activation were hyperbolic and revealed an $Mn^{2+}$-$Mg^{2+}$ interaction.

The conditions under which the effect is seen would be expected to shed light on the mechanism involved. That the $Mn^{2+}$-induced shift in set-point was sharply reduced or even abolished in the absence of $Mg^{2+}$, together with the lesser sensitivity of the $Sr^{2+}$-induced redistribution of $Ca^{2+}$ toward $Mg^{2+}$, are consistent with the known effects of $Mg^{2+}$ on the uptake and release of $Ca^{2+}$ by mitochondria. Under steady-state conditions, the distribution of $Ca^{2+}$ across the uniporter is maintained away from thermodynamic equilibrium by simultaneous efflux through separate pathways [1]. Assuming a constant efflux, the steep relation between $Ca^{2+}$ influx ($v$) and free cation in the medium implies that a change in the former will affect the $Ca^{2+}$ distribution, and hence the mitochondrial set-point, relatively little. However, by reducing the slope of the $v$-versus-free $Ca^{2+}$ curve through inhibition of the uniporter with $Mg^{2+}$, net $Ca^{2+}$ distribution across the membrane and mitochondrial set-point will be made more responsive to changes in $v$. Thus, $Mn^{2+}$ stimulation of $Ca^{2+}$ influx is more apparent in the presence of $Mg^{2+}$. In contrast, the inhibition of $Ca^{2+}$ efflux by $Sr^{2+}$ [5] leads to changes in mitochondrial set-point less sensitive to $Mg^{2+}$. That $Sr^{2+}$ itself is not transported to any significant extent on the $Ca^{2+}$-efflux pathways [5] explains why in spite of its being generally accepted as a useful model for study of $Ca^{2+}$ interaction with the uniporter we were unable to distinguish effects of $Mn^{2+}$ on the steady-state distribution of $^{89}Sr$ across the membrane. The very close approach of the $Sr^{2+}$ distribution to thermodynamic equilibrium that would

be predicted under these conditions meant that any effect of $Mn^{2+}$ on uniporter activity was below our limits of detection.

*The $Mn^{2+}$-$Mg^{2+}$ interaction*

The role of $Mg^{2+}$ in the $Mn^{2+}$ effect seems complex but does not include gross effects on membrane surface charge, since replacement of $Mg^{2+}$ by spermine greatly reduced the subsequent alteration of set-point by $Mn^{2+}$. In any case, such an unspecific mechanism is unlikely, as $Mn^{2+}$ was effective at concentrations as low as 0.5 $\mu$M, or 0.5 nmol/mg mitochondrial protein. Activation of $Ca^{2+}$ influx by $Mn^{2+}$ was saturable and its apparent kinetic parameters were sensitive to $Mg^{2+}$ (Fig. 6). Relatively larger effects were elicited by $Mn^{2+}$ at higher $Mg^{2+}$ levels (Fig. 7), indicating an interaction between the two cations.

Hughes and Exton [6] found that rat liver mitochondria accumulated more $^{45}Ca$ in the presence of 40 $\mu$M $Mn^{2+}$ and at least 0.5 mM $Mg^{2+}$ than they did in the absence of added $Mn^{2+}$ and $Mg^{2+}$. These authors concluded that rather than interfering with $Mg^{2+}$ inhibition of the uniporter, $Mn^{2+}$ must bind in a $Mg^{2+}$-dependent way to distinct activator sites. However, both in experiments using $^{45}Ca$ and using a $Ca^{2+}$ electrode we have been unable to measure an uptake of $Ca^{2+}$ by rat liver mitochondria which significantly exceeded control values. We cannot therefore join Hughes and Exton in excluding the $Mn^{2+}$-$Mg^{2+}$ antagonism mechanism in favour of the more complex $Mn^{2+}$-activation model. Indeed, that $Mg^{2+}$ changed the $K'_m$ and $V_{max}$ of the $Mn^{2+}$ effect on influx (Fig. 6A) and made the kinetics more hyperbolic (Fig. 6B) implies a mixed-type competition between the two cations in modulating uniporter activity. Our results are consistent with an inhibition by extramitochondrial $Mn^{2+}$ of $Mg^{2+}$ effects on the uniporter, analogous to the $Mn^{2+}$-$Mg^{2+}$ antagonism observed in several enzymes. The rather similar concentration dependence of $Mg^{2+}$ inhibition of the uniporter and of $Mg^{2+}$ potentiation of the $Mn^{2+}$ effect on $Ca^{2+}$ influx also suggest a link between the two processes.

*Significance of the phenomenon at a cellular level*

$Mn^{2+}$ directly affects the activity of several key enzymes including superoxide dismutase (EC 1.15.1.1) [14], phospho*enol*pyruvate carboxykinase (EC 4.1.1.32) [15] and pyruvate carboxylase (EC 6.4.1.1) [16]. The experiments reported here show that $Mn^{2+}$ also stimulates $Ca^{2+}$ flux throughout the uniporter in the mitochondrial inner membrane. The only other condition whereby the mitochondrial set-point may be modulated by an activation of the uniporter of which we are aware is that measured after $\alpha$-adrenergic stimulation of heart cells [17]. However, apart from the observation that free $Mn^{2+}$ in hepatocytes from fed rats is almost 3-fold higher than in those from fasted rats [12], little is known of the range over which cytosolic $Mn^{2+}$ varies, and under what conditions. Nor is it clear to what extent transport of $Mn^{2+}$ itself by the uniporter is involved in maintaining the intracellular distribution of the cation. Nevertheless, the finding that physiological concentrations of $Mn^{2+}$ alter the mitochondrial set-point for $Ca^{2+}$ raises the interesting possibility that the uniporter acts as a transducer of $Mn^{2+}$ or $Mn^{2+}$-$Mg^{2+}$ signals into metabolically significant changes in the distribution of $Ca^{2+}$ between cytosol and matrix.

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## References

1 Nicholls, D and Åkerman, K. (1983) Biochim Biophys Acta 683, 57–88
2 Nicholls, D.G (1978) Biochem J 176, 463–474
3 Carafoli, E (1965) Biochim. Biophys. Acta 97, 99–106
4 Ernster, L., Nakazawa, T and Nordenbrand, K. (1978) in The Proton and Calcium Pumps (Azzone, G.F. et al., eds), pp. 163–176, Elsevier/North Holland, Amsterdam
5 Saris, N.-E.L. and Bernardi, P (1983) Biochim Biophys. Acta 725, 19–24
6 Hughes, B.P. and Exton, J H (1983) Biochem J. 212, 773–782
7 Luft, J H. (1971) Anat. Rec. 171, 347–368
8 Scarpa, A. (1979) Methods Enzymol. 56, 301–338
9 Addink, A.D F., Boer, P., Wakabayashi, T. and Green, D.E. (1972) Eur. J Biochem. 29, 47–59
10 Gornall, A G., Bardawill, C J and David, M M (1949) J Biol. Chem 177, 751–766

11 Bragadin, M., Pozzan, T and Azzone, G F (1979) Biochemistry 18, 5972–5978
12 Ash, D E. and Schramm, V.L. (1982) J. Biol. Chem. 257, 9261–9264
13 Åkerman, K.E O. (1977) J. Bioenerg Biomembranes 9, 65–72
14 Weisiger, R.A. and Fridovich, I. (1973) J. Biol Chem. 248, 3582–3592
15 Utter, M.F. and Kolenbrander, H.M. (1972) in The Enzymes (Boyer, P D., ed ), Vol 6, pp 117–168, Academic Press, New York
16 McClure, W R., Lardy, H A. and Kneifel, H.P. (1971) J. Biol. Chem. 246, 3569–3578
17 Crompton, M., Kessar, P. and Al-Nasser, I. (1983) Biochem J. 216, 333–342