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Effects of spermine on mitochondrial Ca^{2+} transport and the ranges of extramitochondrial Ca^{2+} to which the matrix Ca^{2+}-sensitive dehydrogenases respond

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1. Spermine has previously been reported to be an activator of mitochondrial Ca^{2+} uptake [Nicchitta & Williamson (1984) J. Biol. Chem. **259**, 12978–12983]. This is confirmed in the present studies on rat heart, liver and kidney mitochondria by using the activities of the Ca^{2+}-sensitive intramitochondrial dehydrogenases (pyruvate, NAD^+–isocitrate and 2-oxoglutarate dehydrogenases) as probes for matrix Ca^{2+}, and also, for the heart mitochondria, by using entrapped fura-2. 2. As also found previously [Damuni, Humphreys & Reed (1984) Biochem. Biophys. Res. Commun. **124**, 95–99], spermine activated extracted pyruvate dehydrogenase phosphate phosphatase. However, it was found to have no effects at all on the extracted NAD^+–isocitrate or 2-oxoglutarate dehydrogenases. It also had no effects on activities of the enzymes in mitochondria incubated in the absence of Ca^{2+}, or on the Ca^{2+}-sensitivity of the enzymes in uncoupled mitochondria. 3. Spermine clearly activated ^{45}Ca uptake by coupled mitochondria, but had no effect on Ca^{2+} egress from mitochondria previously loaded with ^{45}Ca. 4. Spermine (with effective K_m values of around 0.2–0.4 mM) caused an approx. 2–3-fold decrease in the effective ranges of extramitochondrial Ca^{2+} in the activation of the Ca^{2+}-sensitive matrix enzymes in coupled mitochondria from all of the tissues. The effects of spermine appeared to be largely independent of the other effectors of mitochondrial Ca^{2+} transport, such as Mg^{2+} (inhibitor of uptake) and Na^+ (promoter of egress). 5. In the most physiological circumstance, coupled mitochondria incubated with Na^+ and Mg^{2+}, the presence of saturating spermine (2 mM) resulted in an effective extramitochondrial Ca^{2+} range for matrix enzyme activation of from about 30–50 nM up to about 800–1200 nM, with half-maximal effects around 250–400 nM-Ca^{2+}. The implications of these findings for the regulation of matrix and extramitochondrial Ca^{2+} are discussed.

INTRODUCTION

The Ca^{2+}-transport system of the inner membrane of mammalian mitochondria consists of an electrophoretic Ca^{2+} uniporter for uptake into the matrix driven by the membrane potential (about 180 mV, negative inside) set up through proton extrusion by the respiratory chain, and probably two egress mechanisms, the predominant of which is an electroneutral Na^+/Ca^{2+} exchanger that is ultimately also driven by the protonmotive gradient through subsequent Na^+/H^+ exchange (for reviews see, e.g., Nicholls & Akerman, 1982; Crompton, 1985; Carafoli, 1987); there is also a less well-characterized Na^+-independent egress mechanism which may involve direct $Ca^{2+}/2H^+$ exchange. The Ca^{2+} uniporter can be inhibited physiologically by Mg^{2+} and also artificially by Ruthenium Red, whereas the Na^+/Ca^{2+} egress mechanism can be inhibited physiologically by increases in extramitochondrial Ca^{2+}, and also artificially by drugs such as diltiazem, which are more normally used as blockers of the plasma-membrane Ca^{2+} channel (see the above references).

There are two major functions, which are likely to be mutually exclusive, which have been proposed for this Ca^{2+}-transport system. The earlier, though still advocated (e.g. Chiesi *et al.*, 1988), of these is that it plays a key role in buffering or setting the extramitochondrial (i.e. cytoplasmic) concentration of Ca^{2+} (see, e.g., Fiskum & Lehninger, 1982; Akerman & Nicholls, 1983). For this property to be exhibited, the egress pathways for Ca^{2+} have to be saturated, and this requires in excess of approx. 10–15 nmol of total Ca content per mg of mitochondrial protein (see the Discussion section). The alternative proposal is that its primary function is rather to regulate matrix Ca^{2+} concentration and relay changes in cytoplasmic Ca^{2+} into this compartment (see, e.g., Denton & McCormack, 1980, 1985; Hansford, 1985) and hence regulate oxidative metabolism, as there are three key Ca^{2+}-sensitive intramitochondrial dehydrogenases, namely the pyruvate (PDH), NAD^+–isocitrate (NAD-ICDH) and 2-oxoglutarate (OGDH) dehydrogenases. Thus it is envisaged that hormones and other agents which promote energy-requiring events such as contraction and secretion by raising cytosolic Ca^{2+} would also, as a result, promote energy production (see the above references). In the presence of physiological Na^+ and Mg^{2+}, activation of these enzymes can be demonstrated within intact coupled mitochondria from a variety of different mammalian sources as extramitochondrial Ca^{2+} is raised within the expected physiological

Abbreviations used: PDH, pyruvate dehydrogenase complex; PDH_a, the active, non-phosphorylated, form of PDH; OGDH, 2-oxoglutarate dehydrogenase complex; NAD-ICDH, NAD^+–isocitrate dehydrogenase (EC 1.1.1.41); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $K_{0.5}$ value, the concentration of effector required for half-maximal response.

range (i.e. approx. 0.05–2 μM; see Denton & McCormack, 1985) and as total Ca content rises in the approximate range 0–4 nmol/mg of protein. It was therefore proposed (e.g. Denton & McCormack,1985; Hansford, 1985) that the ability of mitochondria to buffer extramitochondrial [Ca^{2+}] may be reserved for more pathophysiological circumstances where there is an abnormal influx of Ca^{2+} across the plasma membrane, resulting in the capacity of the normal cellular Ca^{2+}-buffering systems being exceeded.

Nicchitta & Williamson (1984) reported that the polyamine spermine is a potent activator of mitochondrial Ca^{2+} uptake; this has since been confirmed by Lenzen *et al.* (1986) and Kroner (1988), though earlier Akerman (1977) had reported that it inhibited this process. In the studies by Nicchitta & Williamson (1984) and Lenzen *et al.* (1986), it was clearly demonstrated that spermine could thus lower the set-point at which the mitochondria buffer the extramitochondrial Ca^{2+} environment to the sub-micromolar range. Given these findings and the above controversies, it seemed important to establish the effects of spermine on the ranges of extramitochondrial Ca^{2+} to which the matrix Ca^{2+}-sensitive dehydrogenases would exhibit Ca^{2+} regulation under different conditions. A small part of the present work has been previously reported briefly as a meeting abstract (McCormack, 1987).

MATERIALS AND METHODS

All biochemicals and chemicals used in this study were of the highest grade commercially available and were purchased from either Sigma Chemical Co. (Poole, Dorset, U.K.) [including spermine (diphosphate salt)], BDH Chemicals (Poole, Dorset, U.K.), or Boehringer Corp. (Lewes, East Sussex, U.K.), except for *p*-(*p*-aminophenyl)-azobenzenesulphonic acid (used in the PDH assay; see below) which was from Pfaltz and Bauer, Stanford, CT 06902, U.S.A., diltiazem {*cis*-(+)-3-acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one)}, which was kindly given by Dr. G. Satzinger of Goedecke A. G., Freiburg, Germany, and fura-2 and its acetoxymethyl ester, which were from Calbiochem (Cambridge, U.K.). The radioisotopes used were from Amersham International (Amersham, Bucks., U.K.).

Mitochondria were prepared from rat heart (Denton *et al.*, 1980), liver (McCormack, 1985*a*) and kidney (McCormack *et al.*, 1988) after Polytron homogenizations of the tissues as described in the appropriate reference; the liver and kidney preparations included a purification step through a Percoll gradient to remove endoplasmic-reticulum contamination, in particular (Assimacopoulos-Jeannet *et al.*, 1986). Mitochondria were incubated (at approx. 0.5–2 mg of protein/ml) at 30 °C in a basic medium consisting of 120 mM-KCl, 20 mM-Tris and 5 mM-KH_2PO_4 (pH 7.3) together with appropriate amounts of EGTA–Ca buffers to generate the required extramitochondrial Ca^{2+} concentrations (see McCormack, 1985*b*), and other additions as indicated in legends. Potassium salts were used throughout unless indicated. The addition of extra phosphate alone (as spermine diphosphate was used) had no effects on the results obtained. Where appropriate, rat heart mitochondria were loaded with fura-2 as fully described in McCormack *et al.* (1989), which also details how the Ca^{2+}-dependent changes in fluorescence were monitored and calibrated.

The Ca^{2+}-sensitive properties of the PDH system and of OGDH and NAD-ICDH were assayed for within the intact heart (Denton *et al.*, 1980), liver (McCormack, 1985*a*,*b*) and kidney (McCormack *et al.*, 1988) mitochondria as described previously. The extraction of mitochondria and the assay of the Ca^{2+}-sensitive enzymes were also as described previously: PDH phosphate phosphatase (Marshall *et al.*, 1984), NAD-ICDH (Denton *et al.*, 1978) and OGDH (McCormack & Denton, 1979). A unit of enzyme activity is the amount that catalyses the conversion of 1 μmol of substrate/min at 30 °C.

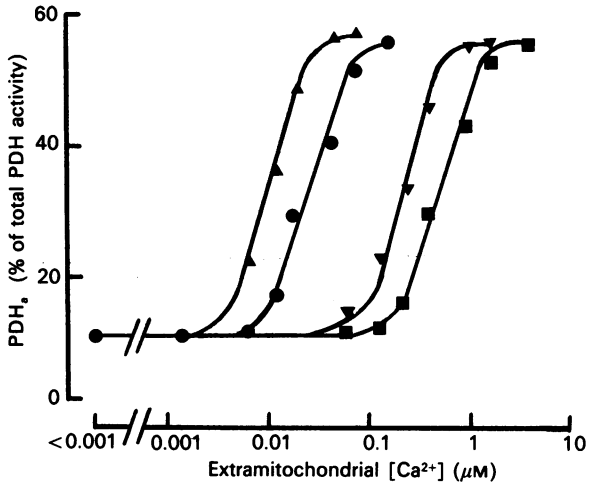

Fig. 1. Effects of spermine on the Ca^{2+}-dependent activation of PDH in coupled rat heart mitochondria incubated with or without Na^+ and Mg^{2+}

Rat heart mitochondria (approx. 0.5 mg of protein/ml) were incubated for 4 min at 30 °C in KCl-based medium (see the Materials and methods section) containing 10 mM-2-oxoglutarate, 0.1 mM-L-malate and EGTA–Ca buffers (at 5 mM-EGTA) to give the concentrations of free extramitochondrial Ca^{2+} shown, and with other additions as follows: none (●), 0.5 mM-spermine (▲), 10 mM-NaCl plus 2 mM-$MgCl_2$ (■), 10 mM-NaCl plus 2 mM-$MgCl_2$ plus 0.5 mM-spermine (▼). PDH_a and total PDH activities were measured as described in the Materials and methods section [see Denton *et al.* (1980) for full details; total activity was unaffected by spermine, and was 101±6 munits/mg protein (mean±S.E.M. for 24 observations)]. Similar results were obtained after incubations for 8 min, and essentially similar results could be obtained with liver or kidney mitochondria (see Table 1), except that with the former some pyruvate (1 mM) also had to be added (see McCormack, 1985*b*). ATP content was unaffected by spermine or by any of the other conditions over the ranges of Ca^{2+} shown, and averaged 8.2±0.3 (52) nmol/mg of protein. However, it should be noted that, if these Ca^{2+} ranges were exceeded, then there were apparent time-dependent diminutions of mitochondrial ATP content, as has been noted previously, and which are presumably the result of saturation of Ca^{2+} egress and resultant net Ca^{2+} accumulation (to a great extent from the EGTA–Ca buffer at 5 mM-EGTA), leading to generalized perturbation of mitochondria (see McCormack, 1985*b*). Each point represents the mean of observations made on 3–5 different preparations.

Mitochondria were loaded with ^{45}Ca (but with total Ca loads within the Ca^{2+}-regulatory ranges of the enzymes) as described by McCormack & Denton (1984) (heart) and McCormack (1985*c*) (liver) for subsequent studies on Ca^{2+} egress, which were carried out as described in the above references. Mitochondrial total Ca content was measured as described by Assimacopoulos-Jeannet *et al.* (1986) and mitochondrial protein was measured by the method of Gornall *et al.* (1949). Calculations were carried out as detailed in McCormack (1985*b*); statistical significance was assessed by Student's *t* test.

RESULTS

The major advantages of using the intramitochondrial Ca^{2+}-sensitive enzymes as probes for matrix Ca^{2+} are that this allows the monitoring of changes in Ca^{2+} fluxes and steady-state Ca^{2+} distributions across the inner membrane to be undertaken while the mitochondria are incubated with buffered and low concentrations of Ca^{2+} within the physiological range and at physiological Ca loads (see Denton & McCormack, 1985), i.e. to match most closely physiological circumstances. These advantages are also afforded by fura-2-loaded rat heart mitochondria (see McCormack *et al.*, 1989). This is in contrast with other techniques which have been used to monitor the effects of spermine on these parameters (Nicchitta & Williamson, 1984; Lenzen *et al.*, 1986), which principally used external Ca^{2+} electrodes or indicators to monitor changes in the extramitochondrial concentration of Ca^{2+} set by the mitochondria (i.e. when the egress pathways are saturated).

A concentration of 0.5 mM-spermine was chosen for most of the experiments. This was partly due to spermine solubility in stock solutions, but largely because the reported $K_{0.5}$ values for its effects on Ca^{2+} uptake were 170 μM (Nicchitta & Williamson, 1984) and 50 μM (Lenzen *et al.*, 1986), with both reporting saturation at about 0.4 mM. However, Kroner (1988) reported a $K_{0.5}$ value of around 0.2 mM, which is closer to that found here (see below). Nevertheless, 0.5 mM is certainly close to saturating (Fig. 5, below). Also in common with these previous reports, spermidine was found to be much less effective (5–10 times) than spermine, and putrescine was ineffective (results not shown).

Fig. 1 and Table 1 show that 0.5 mM-spermine produced marked decreases in the $K_{0.5}$ values for extramitochondrial Ca^{2+} in the Ca^{2+}-dependent activations of PDH in fully coupled mitochondria prepared from rat heart, kidney or liver. Fig. 2 and Table 1 show that very similar effects of 0.5 mM-spermine were evident on the Ca^{2+}-dependent activations of OGDH under similar circumstances. No techniques are yet available to assess the Ca^{2+}-sensitivity of NAD-ICDH in intact rat heart mitochondria, for reasons discussed previously (Denton *et al.*, 1980), and there are also data-interpretation problems with assessing the Ca^{2+}-sensitivity of this enzyme in liver (McCormack, 1985*b*) and kidney (McCormack *et al.*, 1988) mitochondria, largely because there is as yet no satisfactory way of eliminating subsequent flux through OGDH. There are also reports that this enzyme may respond to a higher Ca^{2+}-concentration range, of up to an order of magnitude, than PDH and OGDH (Rutter & Denton, 1988). However, bearing these provisos in mind, the data of Fig. 3 and Table 1 suggest that spermine has very similar effects on the Ca^{2+}-sensitivity of this enzyme to changes in extramitochondrial Ca^{2+} as it has on PDH and OGDH, at least in kidney and liver mitochondria. Rutter & Denton (1988) also showed that the Ca^{2+}-sensitivity of NAD-ICDH and OGDH may be altered by changes in the ATP/ADP ratio; however, spermine (0.5 mM) was found not to bring about any changes in mitochondrial ATP content [assayed as in Denton *et al.*, 1980) and McCormack (1985*b*)] in the present study (see Fig. 1 legend). It should also be noted that the O_2 electrode can be used to monitor Ca^{2+}-dependent effects on OGDH in heart (Denton *et al.*, 1980) and kidney (McCormack *et al.*, 1988) mitochondria, and on NAD-ICDH in kidney

Table 1. Effects of spermine (0.5 mM) on the $K_{0.5}$ values for extramitochrondrial Ca^{2+} in the Ca^{2+}-dependent activations of the matrix Ca^{2+}-sensitive dehydrogenases within intact mitochondria from rat heart, liver and kidney incubated under different conditions

Data from various experiments of the types shown in Figs. 1–3 have been combined to give the $K_{0.5}$ values ± S.D. shown; values were derived from 20–50 observations over suitable [Ca^{2+}] ranges, which were made on at least three different preparations of mitochondria in each case: n.d., not determined. For the experiments with uncoupler for PDH measurements, 2 mM-MgATP was present, together with oligomycin (5 μg/ml) and rotenone (0.2 μg/ml).

	$K_{0.5}$ values (nM) for extramitochondrial Ca^{2+} in the activation of rat:							
	Heart		Liver			Kidney		
Condition	PDH	OGDH	PDH	OGDH	NAD-ICDH	PDH	OGDH	NAD-ICDH
Control	35±2	29±3	114±9	129±8	142±12	28±3	22±1	38±2
0.5 mM-Spermine	15±1	16±1	31±2	33±2	29±3	10±1	11±1	15±1
10 mM-NaCl	133±16	92±6	n.d.	201±20	n.d.	n.d.	114±10	n.d.
Na^+, spermine	72±9	41±4	n.d.	60±10	n.d.	n.d.	30±3	n.d.
2 mM-$MgCl_2$	192±20	149±14	402±16	356±21	n.d.	184±6	149±5	n.d.
Mg^{2+}, spermine	74±6	66±6	106±8	126±12	n.d.	49±4	68±3	n.d.
Na^+, Mg^{2+}	551±36	504±32	586±27	553±36	602±42	441±18	392±21	504±20
Na^+, Mg^{2+}, spermine	295±12	266±25	333±20	315±16	350±27	195±6	242±12	300±21
1 μM-FCCP	1110±56	966±29	1249±40	n.d.	n.d.	n.d.	946±41	1006±24
FCCP, spermine	975±32	930±17	1066±51	n.d.	n.d.	n.d.	971±26	1145±40

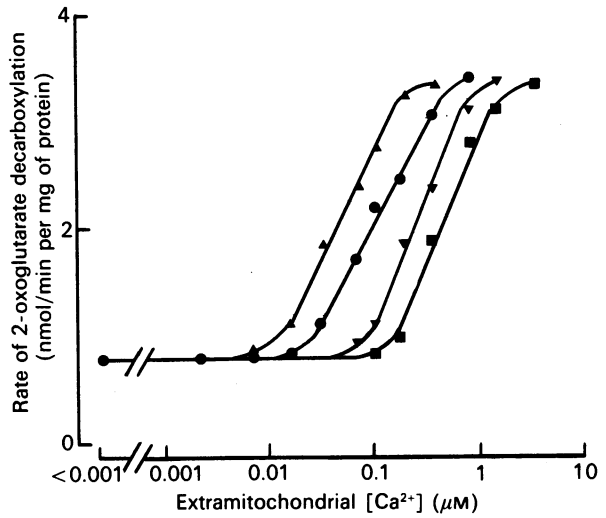

Fig. 2. Effects of spermine on the Ca^{2+}-dependent activation of OGDH in coupled rat liver mitochondria incubated with or without Na^+ and Mg^{2+}

Rat liver mitochondria (approx. 0.5 mg of protein/ml) were incubated at 30 °C in 200 μl of KCl-based medium (see the Materials and methods section) containing 0.5 mM-L-malate and EGTA–Ca buffers (at 5 mM-EGTA) to give the extramitochondrial concentrations of Ca^{2+} shown, in a small test tube within a sealed vial (the latter containing 0.5 ml of 2-phenethylamine), and in the presence of: no further additions (●), 0.5 mM-spermine (▲), 10 mM-NaCl plus 2 mM-$MgCl_2$ (■), 10 mM-NaCl plus 2 mM-$MgCl_2$ plus 0.5 mM-spermine (▼) [see McCormack (1985*a*) for full details]. After 5 min preincubation, 50 μM-2-oxoglutarate was added in volume of 5 μl and containing a suitable amount of 2-oxo[1-^{14}C]glutarate, followed 2.5 min later by 50 μl of 20% (v/v) $HClO_4$. Values shown are means of observations made on 3–5 different preparations. Spermine had no effect on maximal rates of 2-oxoglutarate decarboxylation (at 2 mM-2-oxoglutarate) (results not shown), and essentially similar results could be obtained by using this technique with either rat heart or kidney mitochondria (see Table 1).

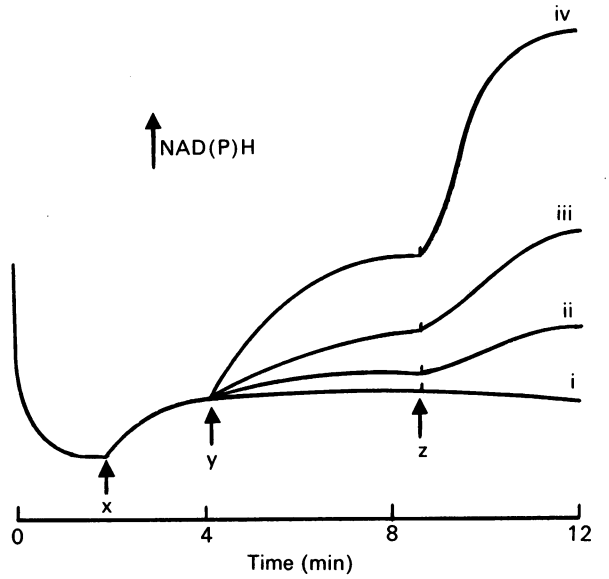

Fig. 3. Effects of spermine on the Ca^{2+}-dependent activation of NAD-ICDH in coupled rat kidney mitochondria

Rat kidney mitochondria (approx. 1 mg of protein/ml) were incubated [with stirring in a cuvette in a fluorimeter with wavelength settings of 340 nm (excitation) and 460 nm (emission)] at 30 °C and for the times shown, in KCl-based medium (see the Materials and methods section) containing initially 2 mM-EGTA and 0.25 mM-hydroxymalonate [see McCormack *et al.* (1988) for full details]. The following additions were then made, as indicated: at arrow x, 100 μM-*threo*-D_S-isocitrate; at arrow y, (i) 1 mM-EGTA, (ii) 0.25 mM-EGTA plus 0.25 mM-$CaCl_2$ (as a buffer solution, to give a resultant free extramitochondrial Ca^{2+} concentration of approx. 7 nM), (iii) 0.5 mM-EGTA plus 0.5 mM-$CaCl_2$ (13 nM), (iv) 1 mM-EGTA plus 1 mM-$CaCl_2$ (26 nM); at arrow z, 0.5 mM-spermine. *threo*-D_S-Isocitrate-induced NAD(P)H production was monitored as indicated. A typical trace is shown; this was repeated on at least three different preparations. If spermine was present initially (i.e. before the Ca^{2+}), then the same resultant NAD(P)H levels were achieved [although rates of NAD(P) reduction were, of course, slightly faster]. Spermine had no effects on maximal NAD(P)H production (at 5 mM-*threo*-D_S-isocitrate). Essentially similar results could be obtained with liver mitochondria for NAD-ICDH, or with heart or liver mitochondria if OGDH activity was assayed in a similar manner [but with 2-oxoglutarate with malonate (not shown, but see Table 1)].

mitochondria, in the presence of ADP or uncoupler; similar results to those shown in Figs. 1–3 and Table 1 were obtained with this technique (not shown).

Control experiments established that spermine had no effects at all on any of the kinetic parameters (including Ca^{2+}-sensitivities) of OGDH and NAD-ICDH in extracts of mitochondria. Spermine was also found not to affect the sensitivity of the free-acid form of fura-2 to Ca^{2+} (see below). The observations by Damuni *et al.* (1984) and Thomas *et al.* (1986) that spermine activated extracted PDH phosphate phosphatase by decreasing its $K_{0.5}$ value for Mg^{2+} in both the presence and the absence of Ca^{2+} were confirmed in the present study (results not shown). Damuni & Reed (1987) also reported a spermine-activated bivalent-cation-independent phosphatase from bovine kidney mitochondria which showed some activity towards PDH phosphate. However, spermine had no effects on the amounts of PDH_a [or the activities of OGDH and NAD-ICDH (Figs. 2 and 3)] in intact mitochondria incubated with EGTA alone, and in either the absence (Fig. 1) or the presence (results not shown) of pyruvate, which activates PDH through its inhibition of PDH_a kinase. Spermine also had no effects on the Ca^{2+}-sensitivity of PDH, OGDH or NAD-ICDH in uncoupled mitochondria (Table 1). Also, spermine uptake by mitochondria is very slow (Toninello *et al.*, 1985). Thus, overall it would appear to be entirely valid to use these enzymes' activities (and fura-2) in intact mitochondria to assess the effects of spermine on this Ca^{2+}-transport system.

Figs. 1–3 and Table 1 show that the effects of spermine on the distribution of Ca^{2+} across the inner membrane are evident in both the absence and the presence of either or both Na^+ and Mg^{2+}, and that the effects appear to be of similar magnitude in all of these different conditions (around 2–3-fold).

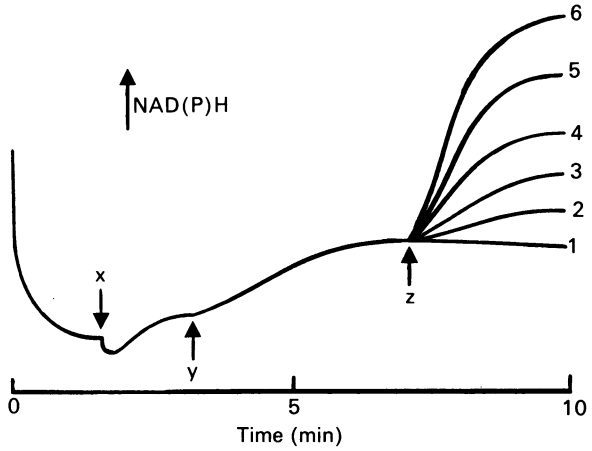

Fig. 4. Effects of increasing spermine concentration on the activation of OGDH within rat liver mitochondria

Rat liver mictochondria were incubated in a fluorimeter exactly as was described in Fig. 3 for kidney mitochondria, except that 0.25 mM-malonate replaced the hydroxymalonate. The following additions were then made as indicated: at arrow x, 100 μM-2-oxoglutarate; at arrow y, 1 mM-EGTA plus 1 mM-$CaCl_2$ (resultant free extramitochondrial $[Ca^{2+}]$ of 26 nM); at arrow z, concentrations of spermine as follows: 1, none; 2, 0.05 mM; 3, 0.1 mM; 4, 0.2 mM; 5, 0.5 mM; 6, 2 mM. A typical experiment is shown; see Fig. 5 and Table 2.

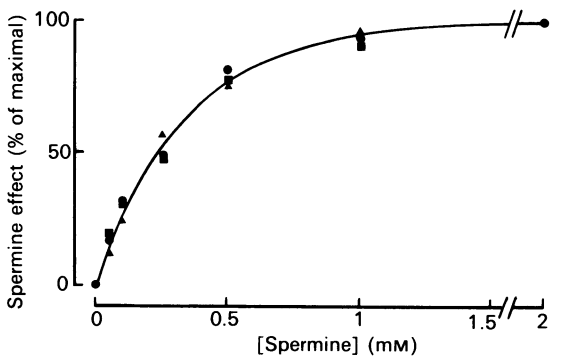

Fig. 5. Concentration-dependency of the effects of spermine on the Ca^{2+}-dependent activations of the Ca^{2+}-sensitive intramitochondrial dehydrogenases within intact rat heart, liver and kidney mitochondria

Data were combined from experiments of the types shown in Figs. 1–3 (but at different [spermine]) and Fig. 4, which were carried out on rat heart (●), liver (■) and kidney (▲) mitochondria. Data on the sensitivity to changes in extramitochondrial Ca^{2+} of PDH, OGDH and NAD-ICDH have been combined for each type of mitochondria for clarity, as no significant differences between enzymes, or indeed type of mitochondria, were evident (see Table 2). The presence or absence of Na^+ and/or Mg^{2+} did not substantially affect the results.

Fig. 4 shows the effects of increasing spermine concentrations on the distribution of Ca^{2+} across the inner membrane of rat liver mitochondria as assessed by matrix OGDH, and Fig. 5 and Table 2 show that the concentration dependency appeared to be similar for each type of mitochondria.

Nicchitta & Williamson (1984) reported that, in addition to its promotion of mitochondrial Ca^{2+} uptake, spermine also promoted, though to a lesser degree, Ca^{2+} egress, so that there would be an increased rate of Ca^{2+} cycling across the inner membrane, but that lower set-point values for extramitochondrial Ca^{2+} could be achieved when the mitochondria were in their Ca^{2+}-buffering mode. This was achieved by a decrease in K_m for Ca^{2+} on the egress pathway [although it is unclear, and contrary to that argued in Nicchitta & Williamson (1984), how this change in K_m could affect the set-point (see the Introduction)]. However, Lenzen *et al.* (1986) reported that the addition of spermine after Ruthenium Red and Na^+ resulted in increased Ca^{2+} uptake. This could suggest that spermine inhibits egress, although those authors rather suggested that it was blocking the effects of Ruthenium Red, which is perhaps more likely, as only a low (250 nM) concentration of Ruthenium Red was used. However, evidence for competing effects of spermine and Ruthenium Red was not apparent in the present study. This was not fully explored; however,

Table 2. $K_{0.5}$ values for spermine in its effects on the distribution of Ca^{2+} across the inner membrane of mitochondria from rat heart, liver and kidney

Data from various experiments of the types shown in Figs. 1–3 and 6 (below) (but at different concentrations of spermine) and in Figs. 4 and 5 have been combined to give the $K_{0.5}$ values (±S.D.) shown; each value was derived over suitable [spermine] ranges and is based on 15–30 observations made on at least two different preparations of mitochondria in each case. The presence or absence of Na^+ and/or Mg^{2+} did not appreciably affect the data given below (results not shown): n.d., not determined.

Experimental approach (see other Figs.)	$K_{0.5}$ value (μM) for spermine in causing larger gradients of Ca^{2+} (in:out) across the inner membrane of mitochondria from rat:		
	Heart	Liver	Kidney
PDH, Ca^{2+}-sensitivity (Fig. 1)	246±12	194±20	302±15
OGDH, Ca^{2+}-sensitivity (Figs. 2 and 4)	295±20	324±18	224±21
NAD-ICDH, Ca^{2+}-sensitivity (Fig. 3)	n.d.	n.d.	315±30
Fura-2-loaded (Fig. 6)	251±16	n.d.	n.d.

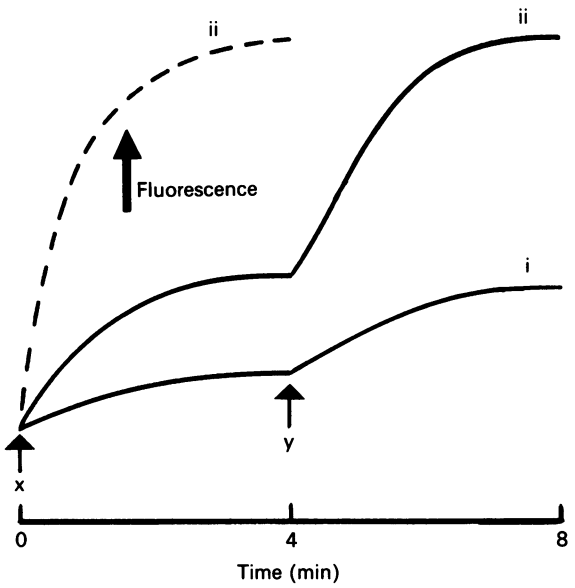

Fig. 6. Effects of spermine on the matrix concentration of Ca^{2+} in fura-2-loaded rat heart mitochondria

Rat heart mitochondria were loaded with fura-2 as described by McCormack *et al.* (1989) and then incubated (at approx. 1 mg of protein/ml) in a stirring cuvette in a fluorimeter (excitation 340 nm, emission 500 nm) in KCl-based medium (see the Materials and methods section) containing 0.5 mM-malonate, 10 mM-2-oxoglutarate (i.e. within the $V_{max.}$ region for OGDH) and 2 mM-EGTA, and in either the initial absence (continuous lines) or presence (dashed line) of 0.5 mM-spermine. After stabilization for 2 min, further additions were made as follows: at arrow x, (i) 0.25 mM-EGTA plus 0.25 mM-$CaCl_2$ (resultant free extramitochondrial $[Ca^{2+}]$ approx. 7 nM), (ii) 0.5 mM-EGTA plus 0.5 mM-$CaCl_2$ (13 nM); at arrow y, 0.5 mM-spermine. A typical experiment is shown; see Table 2 and Fig. 7.

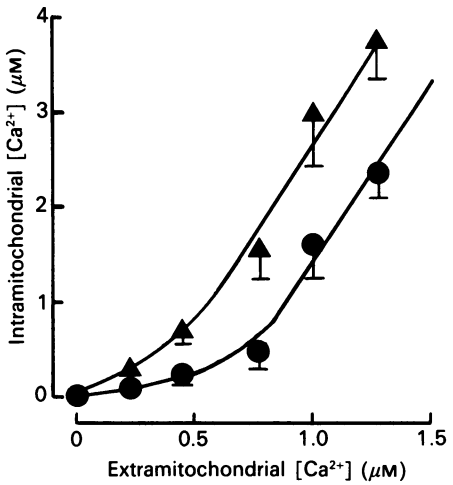

Fig. 7. Effects of spermine on the distribution of Ca^{2+} across the inner membrane of fura-2-loaded rat heart mitochondria incubated with Na^+ and Mg^{2+}

Data were obtained from experiments of the type shown in Fig. 6, but in the additional presence of 10 mM-NaCl and 0.5 mM-$MgCl_2$, and in either the absence (●) or the presence (▲) of 2 mM-spermine. The points and error bars respresent means ± S.E.M. for three different preparations of mitochondria.

Ruthenium Red (2 μM) was found to block all the effects of increasing extramitochondrial Ca^{2+} within the ranges used, in either the absence or the presence of spermine (0.5 mM), and likewise the uptake of ^{45}Ca (see below) (results not shown). Also, Nicchitta & Williamson (1984) reported rather enhanced egress of Ca^{2+} by spermine in the presence of Ruthenium Red; however, again no evidence for this was found in the present study (see below).

The effects of spermine on mitochondrial Ca^{2+} egress were thus explored in the present work by pre-loading mitochondria with ^{45}Ca, but with overall loads of Ca^{2+} still within the enzymes' activatory ranges (around 2–4 nmol/mg of protein), as has been described previously (McCormack, 1985*c*). This means that the Ca loads used in the present study were always lower than those used by Nicchitta & Williamson (1984) (4–60 nmol/mg of protein). No effects at all of spermine were evident on Ca^{2+} egress from rat heart, liver or kidney mitochondria in either the absence or the presence of Na^+ and/or diltiazem, and with or without Ruthenium Red (results not shown), even though losses over the whole range from 20 to 90 % were achieved under the conditions used. This suggests that spermine has no direct effects on mitochondrial Ca^{2+} egress. It should also be noted that spermine caused clear effects on mitochondrial ^{45}Ca uptake in the present investigations when mitochondria were incubated with low or lightly buffered concentrations of Ca^{2+} (results not shown).

The demonstrations that fura-2 can be entrapped into the matrix of rat heart mitochondria (see Davis *et al.*, 1987; Lukacs *et al.*, 1988; McCormack *et al.*, 1989) have allowed another independent means of monitoring spermine effects on Ca^{2+} distribution across the inner membrane of intact mitochondria under physiological incubation conditions. The results of a typical experiment are shown in Fig. 6, and derived $K_{0.5}$ values for extramitochondrial Ca^{2+} and for spermine from such experiments are given in Tables 1 and 2 respectively. Fig. 7 shows the effects of spermine on the distribution of Ca^{2+} across the inner membrane over the range of extramitochondrial $[Ca^{2+}]$ expected in mammalian cells in mitochondria incubated with physiological concentrations of Na^+ and Mg^{2+}. While the present manuscript was in preparation, Moreno-Sanchez & Hansford (1988) published a paper on indo-1-loaded rat heart mitochondria in which some effects of spermine on Ca^{2+} uptake were demonstrated, which correlated with changes in PDH_a.

Fig. 8 shows that spermine did not appear to affect the total Ca content of the mitochondria over the activatory ranges for the enzymes.

DISCUSSION

The study by Nicchitta & Williamson (1984) was on rat liver mitochondria, and Lenzen *et al.* (1986) reported spermine effects on rat liver, heart and brain mitochondria. However, Kroner (1988), although confirming effects on liver, reported no effects on heart. This is clearly at variance with the present work and that of Lenzen *et al.* (1986), which rather suggest that this is likely to be a widespread phenomenon for mitochondria

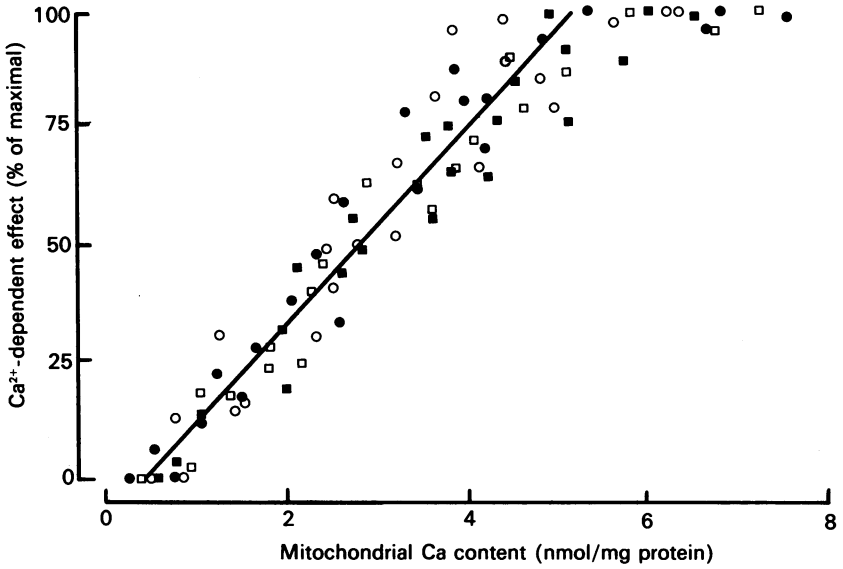

Fig. 8. Effects of spermine on the total Ca content of rat heart and liver mitochondria over the Ca^{2+}-dependent activatory ranges of the intramitochondrial PDH and OGDH

Rat heart (●, ○) or liver (■, □) mitochondria were incubated (at 2–4 mg of protein/ml) at 30 °C in KCl-based medium (see the Materials and methods section) for experiments of the type shown in Figs. 1–3 and in either the absence (●, ■) or the presence (○, □) of 0.5 mM-spermine, and with various concentrations of $CaCl_2$ (0–60 μM) or EGTA so that the full ranges of the Ca^{2+}-dependent effects on PDH or OGDH were observed, and then total Ca contents were measured as described previously (Assimacopoulos-Jeannet *et al.*, 1986). The data from each enzyme were very similar, and have therefore been combined for clarity.

from mammalian tissues. The reasons underlying the negative report on heart by Kroner (1988) remain unknown, except perhaps that albumin was exclusively present in these heart incubations.

The data in the present report clearly indicate that spermine is a potent activator of mitochondrial Ca^{2+} uptake under conditions where the Ca^{2+}-sensitive properties of the matrix dehydrogenases are exhibited; i.e. this property is evident when mitochondria are incubated under conditions where they do not buffer, but rather respond to, extramitochondrial Ca^{2+}, as well as when they are in their buffering mode. Therefore spermine leads to a decrease in the $K_{0.5}$ values for extramitochondrial Ca^{2+} in the activation of the enzymes in coupled mitochondria under all conditions tested (Table 1). The effective extramitochondrial Ca^{2+} ranges, which run from approx. 5-fold lower to about 5-fold higher than the $K_{0.5}$ values, are correspondingly decreased. In the most physiological circumstance, i.e. with both Na^+ and Mg^{2+}, the effective range for each type of mitochondria with spermine was from about 30–50 nM up to about 800–1200 nM, with $K_{0.5}$ values of the order of 250–400 nM. This is still well within the expected cytosolic range for Ca^{2+} (e.g. Carafoli, 1987) and therefore, together with the other supporting evidence (see the Introduction), it is still concluded that the primary function of the Ca^{2+}-transport system is to relay hormonally induced changes in cytoplasmic Ca^{2+} into the matrix for regulation of the dehydrogenases and hence oxidative metabolism. The previous studies by Nicchitta & Williamson (1984) and Lenzen *et al.* (1986) did not test this circumstance (i.e. plus Na^+ and Mg^{2+}), which is therefore most likely to be the explanation for the rather low values of buffering set-point which they report with spermine (0.2–0.3 μM). In the present study, there was a slight indication that spermine may be less effective in the presence of Na^+ and Mg^{2+} (Table 1). As spermine did not appear to affect the total Ca content of the mitochondria over the activatory ranges of the enzymes (Fig. 8), this suggests that spermine does not alter the fact that the two functions of the Ca^{2+}-transport system are still most likely to be mutually exclusive (see the Introduction), and it is the matrix Ca^{2+}-regulatory role which is likely to be the more physiologically relevant. It is also worth noting that the values for total Ca content over the enzymes' activatory range, as opposed to the higher values required for buffering, are much closer to estimates made *in situ* by using X-ray probe microanalysis (e.g. Somlyo *et al.*, 1985; Wendt-Gallitelli, 1986; LeFurgey *et al.*, 1986). These data are thus in some ways contrary to the conclusion of Nicchitta & Williamson (1984) that the effects of spermine on Ca^{2+} egress would allow mitochondria to buffer extramitochondrial Ca^{2+} at lower Ca content; however, it is unclear how a process which depends on egress saturation would be affected by a change in K_m.

The concentrations of spermine used in the present and the previous (see above) studies are within the ranges expected for cellular contents of the polyamine (Pegg, 1986). However, there is no information as to the compartmentation or binding of spermine inside cells, so the actual concentration in the vicinity of the mitochondrial inner membrane is unknown. There therefore remains the intriguing possibility that changes in the cytosolic concentration of spermine may produce significant changes in the relationship between extra- and intra-mitochondrial Ca^{2+}, and that this may contribute to the stimulation of oxidative metabolism

observed in stimulated cells. Relevant to this are reports that the β-adrenergic stimulation of rat kidney (Koenig *et al.*, 1983) and heart (Fan & Koenig, 1988) involves an increase in ornithine decarboxylase activity and a rise in cellular polyamine content.

The interpretation of the existing data on the effects of spermine on Ca^{2+} uptake by mitochondria would suggest that it has a direct effect on the Ca^{2+} uniporter. This suggests that it has an allosteric site on the uniporter (Kroner, 1988) rather than, for example, bringing about its effects through the shielding of other Ca^{2+}-binding sites on the surface of the inner membrane; the results reported here with uncoupler support this. Nor is it likely that the reported effects on Ca^{2+} uptake are due to the general protective effects of spermine on mitochondria, as spermidine appears to behave similarly in this (e.g. Tabor, 1960; Chaffee *et al.*, 1977), and also because the effect of spermine is on the apparent K_m for Ca^{2+} (Nicchitta & Williamson, 1984).

Nicchitta & Williamson (1984) suggested that spermine effects on Ca^{2+} uptake were independent of the effects of Mg^{2+} as an inhibitor of uptake; however, Lenzen *et al.* (1986) reported evidence for interaction of Mg^{2+} and spermine. In the present work spermine did not affect the K_i for Mg^{2+} in its inhibition of Ca^{2+} uptake, nor did Mg^{2+} affect spermine $K_{0.5}$ values (results not shown in full; Table 2). This is in better agreement with Nicchitta & Williamson (1984); the latter, it is suggested, were rather perhaps examining effects of Mg^{2+} on uptake itself, rather than any inhibition of spermine effects.

The differences between the present work and Nicchitta & Williamson (1984) with regard to Ca^{2+} egress is perhaps because in the latter only Na^+-independent egress was monitored, and also that there was some extramitochondrial Ca^{2+} present, which has been shown by Hayat & Crompton (1982) to be an inhibitor of Na^+-dependent Ca^{2+} egress. There thus remains the possibility that spermine may interact with this Ca^{2+}-regulatory site on the Na^+/Ca^{2+} exchanger to bring about the effects noted by Nicchitta & Williamson (1984). Another obvious difference is the large differences in Ca loads of the mitochondria in the two studies (see above).

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