Biochem. J. (1990) 271, 627–634 (Printed in Great Britain)

Measurement of matrix free Mg^{2+} concentration in rat heart mitochondria by using entrapped fluorescent probes

Guy A. RUTTER,*‡ Nicola J. OSBALDESTON,† James G. McCORMACK† and Richard M. DENTON*
*Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.,
and †Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

1. The concentration of free Mg^{2+} ($[Mg^{2+}]_m$) within the matrix of isolated rat heart mitochondria was measured after loading of the mitochondria with the fluorescent Mg^{2+} indicators mag-indo-1 and mag-fura-2. No detectable change in total mitochondrial magnesium content occurred during loading with the indicators. Apparent K_d values for Mg^{2+} of 3.7 mM and 2.3 mM were obtained for mag-indo-1 and mag-fura-2 respectively within mitochondria permeabilized to bivalent cations with ionomycin and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. These values are 2.7- and 1.8-fold greater respectively than those obtained for the free acid forms of the dyes in incubation medium. 2. Based on the above K_d values, mitochondrial matrix Mg^{2+} concentrations were found to lie in the range 0.8–1.5 mM in the absence, or immediately after the addition, of a respiratory substrate. 3. Incubation of mitochondria in the presence of respiratory substrate, but in the absence of external Mg^{2+}, led to a time-dependent decline in $[Mg^{2+}]_m$ to about half the initial values after 5 min. This was accompanied by a fall in the total mitochondrial magnesium content from 12.7 to 7.0 nmol/mg of protein. 4. ADP (0.5 mM), ATP (0.5 mM) or 10 mM-NaCl had no significant effect on the fall in $[Mg^{2+}]$, whereas 1 μM-nigericin blocked, and 0.3 μM-valinomycin accelerated, the fall. 5. External Mg^{2+} concentrations above 1 mM progressively inhibited and reversed the decline in free and total mitochondrial Mg^{2+}.

INTRODUCTION

Mg^{2+} ions are essential for the activity of many intramitochondrial enzymes, including pyruvate dehydrogenase phosphate (PDHP) phosphatase [which dephosphorylates and concomitantly activates the pyruvate dehydrogenase (PDH) complex], NAD^+–isocitrate dehydrogenase and inorganic pyrophosphatase [1]. The first two of these play a crucial role in the control of citrate-cycle activity [1], whereas changes in intramitochondrial PP_i concentrations may play a key role in the control of respiratory-chain activity through the regulation of matrix volume [2,3].

Ca^{2+} ions also regulate the activity of each of these enzymes, causing the stimulation of PDHP phosphatase and NAD^+–isocitrate dehydrogenase, and inhibition of pyrophosphatase [1]. These effects of Ca^{2+} are, however, strongly dependent on the Mg^{2+} concentration. Thus increases in Mg^{2+} concentration in the low-mM range raise the $K_{0.5}$ value (concentration giving half-maximal effects) for Ca^{2+} of NAD^+–isocitrate dehydrogenase [4] and inorganic pyrophosphatase [2,3]. Similarly, since the effect of Ca^{2+} on the activity of PDHP phosphatase is to lower the $K_{0.5}$ value for Mg^{2+} [5–7], increases in Mg^{2+} gradually diminish the stimulation elicited by Ca^{2+}. A fourth intramitochondrial enzyme, the 2-oxoglutarate dehydrogenase complex, is also activated by Ca^{2+} [8]. However, this enzyme does not require added Mg^{2+}, and the effects of Ca^{2+} are Mg^{2+}-independent ([8]; G. A. Rutter & R. M. Denton, unpublished work).

Insulin causes the activation of PDHP phosphatase in adipose tissue, and studies on permeabilized mitochondria indicate that this may be due to a decrease in the $K_{0.5}$ value of the phosphatase for Mg^{2+} by a mechanism which does not involve changes in the mitochondrial concentration of Ca^{2+} [5,9].

These recent observations have thus made the determination of the free Mg^{2+} concentration ($[Mg^{2+}]_m$) within the matrix of intact mitochondria of some importance. Previous estimates [10,11] have relied on the 'null point' approach to measure this parameter. However, this rather indirect method does not allow measurements within fully coupled mitochondria. We have therefore now addressed this problem using two new fluorescent Mg^{2+} indicators, mag-indo-1 and mag-fura-2 [12,13]. These indicators possess the same fluorophores, but different chelating groups, as the Ca^{2+} indicators indo-1 and fura-2. The latter have been successfully loaded into isolated rat heart mitochondria and used to measure intramitochondrial Ca^{2+} concentrations [14–19].

EXPERIMENTAL

Materials

Mag-indo-1 and mag-fura-2 [acetoxymethyl (AM) and tetrapotassium forms] were purchased from Molecular Probes, Eugene, OR, U.S.A. All other chemicals and biochemicals were from sources given previously [15,20].

Methods

Preparation of mitochondria. Rat heart mitochondria were obtained after rapid tissue disruption with a Polytron PT-20 tissue homogenizer as described previously [21]. The final pellets were resuspended at 20–40 mg of mitochondrial protein/ml in 250 mM-sucrose/20 mM-Tris/HCl (pH 7.4)/2 mM-EGTA (0–4 °C).

Loading of mitochondria with mag-indo-1 and mag-fura-2, and fluorescence measurements. Samples of mitochondrial suspension (50 μl) were incubated with various concentrations of the AM forms of the indicators at 30 °C for 2–15 min. The samples

Abbreviations used: $[Mg^{2+}]_m$, free Mg^{2+} concentration within the mitochondrial matrix; PDH, pyruvate dehydrogenase; PDHP, PDH phosphate; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; K_d value, apparent dissociation constant; $K_{0.5}$ value, concentration giving half-maximal effects. Throughout this paper, $[Mg^{2+}]$ and $[Ca^{2+}]$ refer to the free concentrations of these metal ions.

‡ To whom all correspondence should be addressed.

were then immediately transferred into 2 ml of either medium A [125 mM-KCl, 20 mM-Mops/Tris, pH 7.3, 5 mM-KH_2PO_4, 0.5 mM-EGTA, 0.5 mM-*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) (30 °C)] or medium B [125 mM-KCl, 20 mM-Tris/HCl, pH 7.3, 5 mM-KH_2PO_4], plus other additions as given in the Figure and Table legends. Fluorescence was measured on a Perkin–Elmer LS5 spectrofluorimeter equipped with a thermostatically regulated cell holder and magnetic stirrer.

Control of metal ion concentrations and determination of dissociation constants (K_d values). Free and bound concentrations of metal ions and other species were calculated as described elsewhere [6,9,21]. K_d values for the binding of Mg^{2+} and Ca^{2+} to mag-indo-1 and mag-fura-2 were calculated by non-linear-regression analysis [20] of the fluorescence intensity (F) at appropriate single wavelengths (see the Results section) by fitting data to the following expression, which assumes 1:1 binding:

$$F-F_{min.} = F_{max.}/\{1+(K_d/[M^{2+}])\}$$

where $[M^{2+}]$ is the concentration of Mg^{2+} or Ca^{2+} and $F_{min.}$ and $F_{max.}$ are the fluorescence intensities at zero and saturating $[M^{2+}]$.

Measurement of total magnesium content of mitochondria. This was determined by atomic absorption spectroscopy using a Pye Unicam FP9 spectrophotometer. Samples (0.8 ml) of the mitochondrial incubations (see above) were rapidly sedimented (25 s, 10000 *g*) and washed once in 1 ml of 125 mM-KCl/20 mM-Tris/HCl (pH 7.4)/5 mM-KH_2PO_4 (ice-cold). Mitochondrial pellets were incubated with 0.4 ml of 1.0 M-NaOH for 5 min at 70 °C, and diluted to 1.4 ml with 1 mM-EDTA plus 4 mM-$LaCl_3$ before analysis.

Other methods. O_2 uptake by mitochondria was measured with a Clark-type oxygen electrode [21] in medium A (see above) plus 1 mM-EGTA. Mitochondrial ATP content was measured by luciferin/luciferase assay [22] of samples of the mitochondrial incubations acidified with 2% (w/v) $HClO_4$. Mitochondrial protein was measured as described in ref. [23].

Expression of results. Where appropriate, results are given as means ± S.E.M. for the numbers of preparations (n) given in parentheses. Where single experiments are shown, these were all repeated at least 3 times.

RESULTS

Hydrolysis and trapping of dyes within the mitochondrial matrix

The generation of de-esterified Mg^{2+}-sensitive (and Mn^{2+}-quenchable) mag-indo-1 and mag-fura-2 within the matrix of rat heart mitochondria is shown in Fig. 1. At 30 °C, the extent of conversion of mag-fura-2-AM was variable, but reached a maximum of 20–50% after 5 min at both 20 μM and 100 μM concentrations of dye (Fig. 1). The extent of the conversion of mag-indo-1-AM was generally somewhat greater (at least 50%) after 5 min, and increased further at higher dye concentrations. The localization of the hydrolysed dyes within the mitochondrial matrix was confirmed by the observations that: (i) addition of Mn^{2+} in the presence of Ruthenium Red (to inhibit uptake of the ion; see [16]) failed to quench the signal in the absence of ionomycin; (ii) successive sedimentation and resuspension did not lead to any significant diminution of the signal, which could be quenched by Mn^{2+} in the presence of ionomycin and FCCP.

For routine measurements, a loading period of 5 min was chosen, since this minimized the decline in mitochondrial function and loss of adenine nucleotides which can accompany longer loading periods [16]. Thus, whereas the ATP content of unloaded

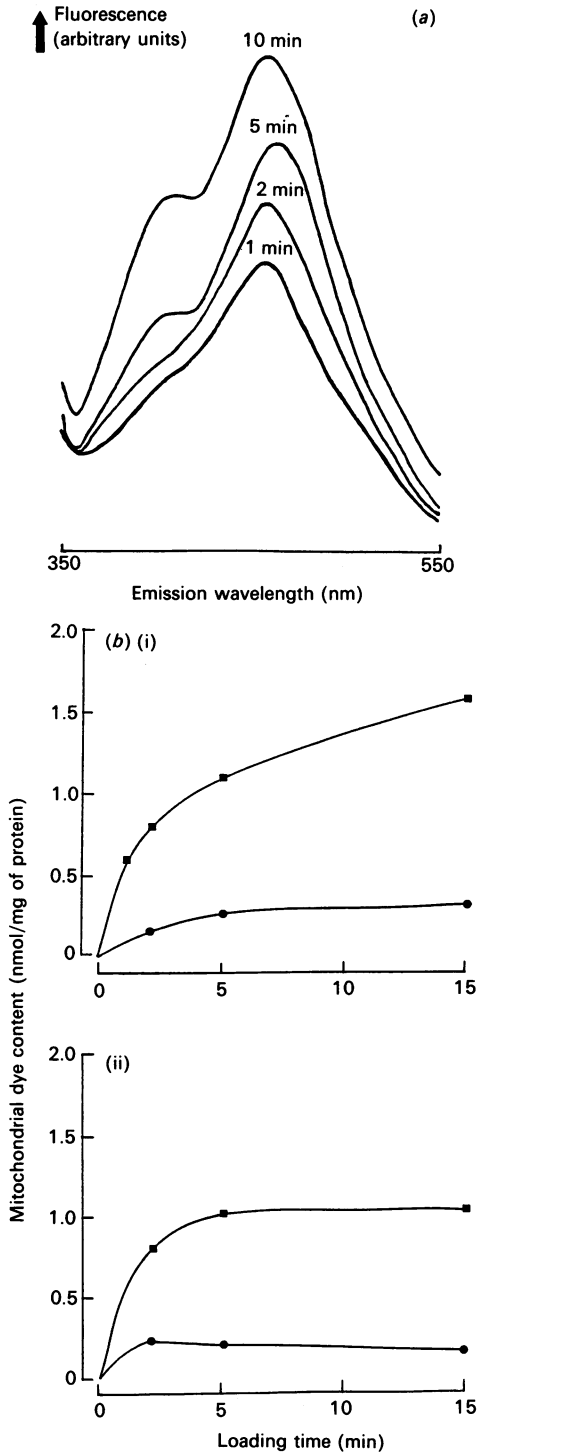

Fig. 1. Generation of hydrolysed mag-indo-1 and mag-fura-2 within the matrix of rat heart mitochondria

(*a*) Emission spectra of mitochondria incubated with 100 μM-mag-indo-1-AM at 30 °C were obtained as described in the Experimental section. The excitation wavelength was 330 nm. Spectra were obtained after incubation for 1, 2, 5 or 10 min (as indicated) in medium A supplemented with 1 μM-FCCP, 2 μM-ionomycin and 50 mM-$MgCl_2$ ($[Mg^{2+}]$ = 44 mM). (*b*) The increase in the intramitochondrial concentration of (i) mag-indo-1 and (ii) mag-fura-2 was determined by experiments of the type described in (*a*). The concentration of the AM forms of the indicators was 20 μM (●) or 100 μM (■). The magnitude of the fluorescence signal (330 nm excitation, 450 nm emission for mag-indo-1; 350 nm excitation, 500 nm emission for mag-fura-2) which was quenched by 2 mM-$MnCl_2$ was related to the concentration of indicators by comparison to standard curves obtained with the pentapotassium salts of the indicators.

mitochondria incubated in the presence of succinate was approx. 3 nmol/mg of protein (after 2 min incubation), comparable values obtained with mitochondria preincubated with the indicators for 2 min or 5 min (at 30 °C) were 1–2 nmol/mg of protein. Furthermore, after the 5 min loading period, the mitochondria remained well coupled (coupling ratios = 10 with unloaded mitochondria and 7 with loaded mitochondria, with 5 mM-2-oxoglutarate as substrate).

Essentially identical calculated values for $[Mg^{2+}]_m$ were obtained after loading mitochondria with either 20 μM or 100 μM of either indicator; however, mitochondria were usually loaded by incubation with 100 μM dye, as this gave the best signal:noise ratio. No loss of total magnesium (measured by atomic absorption) was detectable after dye-loading periods of up to 15 min, nor from mitochondria maintained at 0 °C for up to 24 h (results not shown).

Determination of K_d values of mag-indo-1 and mag-fura-2 within intact mitochondria

Since the estimation of $[Mg^{2+}]_m$ relies crucially on the K_d values of the entrapped indicators, we have endeavoured to establish these precisely with the indicators located within the intact mitochondrial matrix.

With both indicators, calibrations were performed by measuring the fluorescence at a single wavelength (330 nm excitation, 400 nm emission for mag-indo-1, and 312 nm excitation, 500 nm emission for mag-fura-2). Mitochondria were permeabilized to Mg^{2+} (or Ca^{2+}) with the non-fluorescent bivalent-cation ionophore, ionomycin, plus FCCP to eliminate the membrane potential and pH gradient (see Fig. 1) [15–17]. Replacement of FCCP by nigericin, or addition of 1 μM-valinomycin in the presence of FCCP and ionomycin, had no effect on the signals. These observations imply that FCCP plus ionomycin caused the complete collapse of membrane potential and pH gradient, and thus of any gradient of Mg^{2+} across the mitochondrial inner membrane. K_d values obtained after permeabilization with ionomycin plus FCCP are given in Table 1, and compared with those of the free acid forms of the dyes obtained in identical media. Essentially identical values were obtained in experiments where ionomycin was replaced by another bivalent-cation ionophore, bromo-A23187 [16]. The apparent K_d values for the dyes located within the mitochondrial matrix were rather higher (1.5–3.0-fold) than those for the free acids, or those obtained after complete permeabilization of the mitochondria with 0.2% (w/v) Triton X-100. After Triton permeabilization, K_d values for Mg^{2+} of mag-indo-1 and mag-fura-2 were 1.28 ± 0.21 mM ($n = 4$) and 0.74 ± 0.30 mM ($n = 4$) respectively. Since the values obtained after ionomycin/FCCP treatment seem to represent the best estimates of the dissociation constants for the indicators within the environment of the mitochondrial matrix, we have based calculations of $[Mg^{2+}]_m$ on these values.

Some variation was apparent in the measured K_d values for Mg^{2+} between different mitochondrial preparations. This variation was most apparent with mag-indo-1-loaded mitochondria, where K_d values of up to 7 mM were obtained. The K_d value for Mg^{2+} was therefore measured at pH 7.3 for each preparation, and $[Mg^{2+}]_m$ determinations were based on this value; no correction was made for the (weak) pH-dependency of the dyes (Table 1), although it is appreciated that the intramitochondrial pH may be higher than that of the medium in the presence of respiratory substrates [18,19].

Both indicators responded to Ca^{2+} with K_d values in the 10 μM range within the ionomycin/FCCP-permeabilized mitochondria (Table 1). This is rather lower than previously published estimates of the K_d values for Ca^{2+} of mag-fura-2 (around 50 μM; see [12]), and also lower than values observed with the tetrapotassium salts of the indicators in identical media (Table 1). However, interference from Ca^{2+} in the measurement of $[Mg^{2+}]_m$ is unlikely, since, under the conditions employed, mitochondrial $[Ca^{2+}]$ remained at < 0.1 μM when measured with fura-2 or indo-1, or on the basis of the activity of the mitochondrial Ca^{2+}-sensitive dehydrogenases (results not shown; see also [15]). However, these low K_d values for Ca^{2+} precluded the determination of the effects of changes in mitochondrial free Ca^{2+} concentration on $[Mg^{2+}]_m$ (see the Discussion section).

Table 1. Apparent K_d values of mag-indo-1 and mag-fura-2 for Mg^{2+} and Ca^{2+}

Mitochondria were permeabilized to Mg^{2+} by using ionomycin and FCCP as described in the text. All measurements were made in medium A (see the Experimental section). Values within permeabilized mitochondria are the means of observations on three separate preparations. At least eight separate Mg^{2+} concentrations between 0.1 mM and 50 mM were used in each case, and data were fitted to the expression given in the Experimental section. In all cases an excellent fit was obtained, and no systematic trend was apparent in the residuals.

		K_d for Mg^{2+} (mM)		K_d for Ca^{2+} (μM)	
Indicator	pH	Potassium salt	Permeabilized mitochondria	Potassium salt	Permeabilized mitochondria
mag-indo-1	7.3	1.40 ± 0.07	3.77 ± 0.36	22.6 ± 6.0	8.9 ± 2.4
	7.7	1.21 ± 0.14	–	9.5 ± 1.0	–
mag-fura-2	7.3	1.30 ± 0.06	0.25	21.0 ± 1.4	10.3 ± 1.5
	7.7	0.97 ± 0.13	2.48 ± 0.43	13.5 ± 0.9	–

Continuous measurement of $[Mg^{2+}]_m$

Fluorescence spectra obtained with mitochondria loaded with either mag-indo-1 or mag-fura-2 are shown in Fig. 2. $[Mg^{2+}]_m$ was determined from the fluorescence signals either (i) by measurement of the fluorescence at a single excitation and emission wavelength (see above), as used to measure matrix free Ca^{2+} concentrations [15,16], or (ii) from the ratio of the fluorescence intensity at two wavelengths [24–26].

Fig. 3 shows an example of the measurement of $[Mg^{2+}]_m$ at a single wavelength, by using mag-indo-1-loaded mitochondria. Experiments of this type indicated that the addition of an oxidizable substrate led to time-dependent decrease in $[Mg^{2+}]_m$ in the absence of external Mg^{2+}. For example, after the addition of 10 mM-2-oxoglutarate plus 0.5 mM-malonate, $[Mg^{2+}]_m$ fell from an initial value of 0.85 ± 0.1 mM (immediately after substrate addition) to 0.42 ± 0.05 mM after 5 min ($n = 5$). This decline in $[Mg^{2+}]_m$ was completely eliminated in the presence of 1 μM-FCCP or 1 μM-nigericin, but was enhanced by 0.3 μM-valinomycin (Fig. 4). Further, the apparent decrease in $[Mg^{2+}]_m$ was progressively inhibited and reversed by increasing concentrations of external Mg^{2+} [Fig. 4(iv)].

Although allowing excellent time resolution, the measurement of $[Mg^{2+}]_m$ at a single wavelength suffers from the risk of a number of artefacts. For example, changes in mitochondrial volume might affect fluorescence through changes in light scattering. Furthermore, the weaker signal and greater contribution from mitochondrial autofluorescence in measurements using mag-fura-2 mean the single-wavelength approach has limited value when this indicator is used.

We have therefore used fluorescence ratio (R) measurements [24–26] with both indicators to confirm and extend the findings described above. In this case, measurements of the fluorescence at two suitable wavelengths were made either by alternating

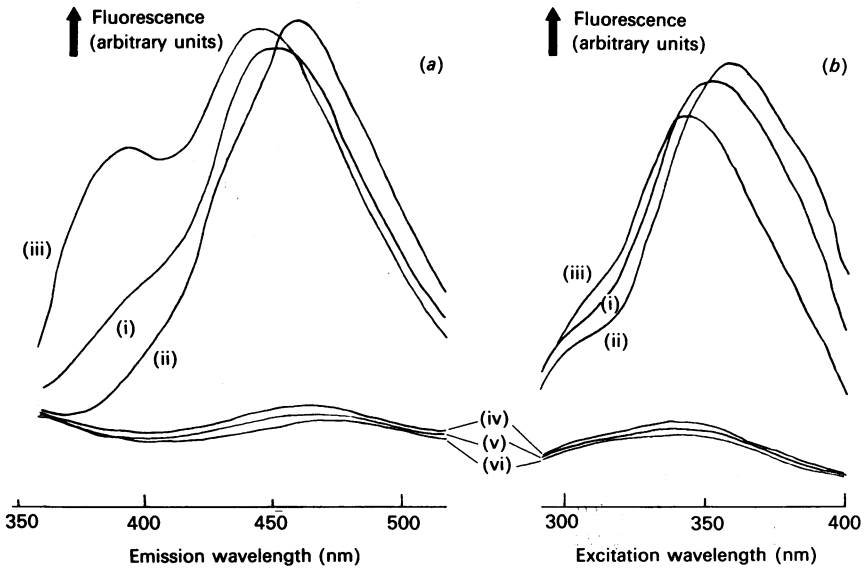

Fig. 2. Measurement of $[Mg^{2+}]_m$ within intact fully coupled mitochondria by using (*a*) mag-indo-1 or (*b*) mag-fura-2

(*a*) Mitochondria were incubated for 5 min at 30 °C either with (i, ii, iii) or without (iv, v, vi) 100 μM-mag-indo-1 AM. Emission spectra were then obtained (see the Experimental section) 1 min after the following additions: (i, iv) 5 mM-Tris succinate; (ii, v) 2 μM-ionomycin plus 1 μM-FCCP; (iii, vi) 50 mM-$MgCl_2$. (*b*) As (*a*), but with mag-fura-2 replacing mag-indo-1 AM. The excitation spectra were obtained at an emission wavelength of 500 nm.

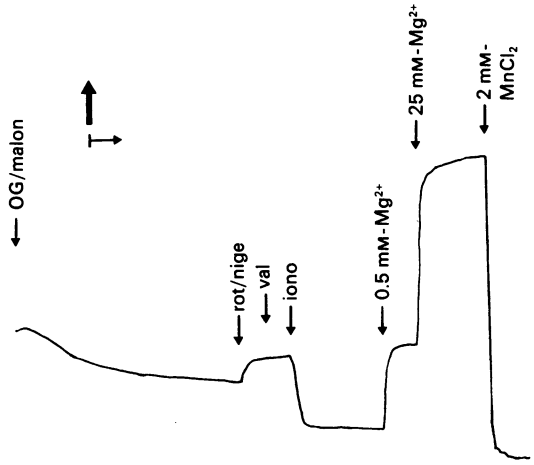

Fig. 3. Continuous monitoring of $[Mg^{2+}]_m$ by using mag-indo-1

Fluorescence (*F*) was measured at 400 nm (330 nm excitation) in mag-indo-1-loaded mitochondria (see the Experimental section) incubated in medium B plus 0.2 mM-EGTA. The bold arrow indicates the direction of increasing fluorescence, and the horizontal arrow, 1 min. Additions were: OG/malon, 10 mM-2-oxoglutarate plus 1 mM-malonate; rot/nige, 1 μM-rotenone plus 1 μM-nigericin; val, 0.3 μM-valinomycin, iono, 2 μM-ionomycin. The increase in fluorescence on addition of rotenone plus nigericin resulted from an increase in NAD(P)H fluorescence (control experiment not shown). Values of $[Mg^{2+}]$ (see the text) were calculated from the fluorescence at zero ($F_{min.}$) and saturating (25 mM) Mg^{2+} ($F_{max.}$), according to:

$$[Mg^{2+}]_m = K_d \cdot (F - F_{min.})/(F_{max.} - F)$$

K_d values for Mg^{2+} were determined as described in the text.

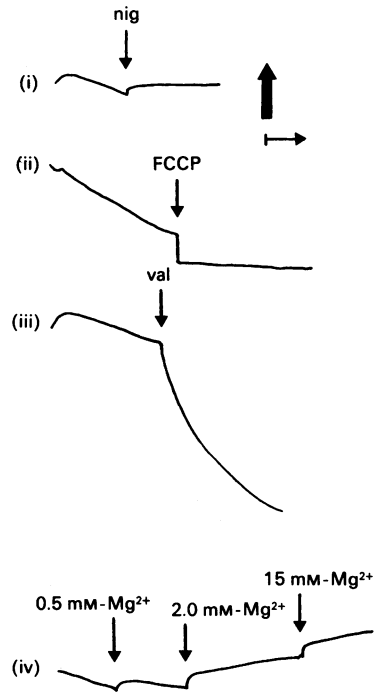

Fig. 4. Effects of ionophores and external Mg^{2+} on changes in $[Mg^{2+}]_m$

Fluorescence of mag-indo-1-loaded mitochondria was measured as in Fig. 3, in the presence of 10 mM-2-oxoglutarate plus 1 mM-malonate. Additions were; (i) nig, 1 μM-nigericin; (ii) 1 μM-FCCP; (iii) val, 0.3 μM-valinomycin; (iv) $MgCl_2$ to give $[Mg^{2+}]$ as shown. The bold arrow corresponds to increasing fluorescence and the light arrow to 1 min.

between these wavelengths up to once every 20 s (Fig. 3*a*), or after obtaining full spectra (see Fig. 2). With mag-indo-1, the emission wavelength pair (values in nm) was either 384 and 506, or 400 and 500, with excitation at 330 nm; with mag-fura-2, an excitation wavelength pair of 312 and 372 (emission at 500 nm) was found optimal. After measurement of K_d, $R_{min.}$ and $R_{max.}$ values, $[Mg^{2+}]_m$ was calculated as described elsewhere [26]. Since variations in $R_{max.}$ as well as K_d values (see above) were apparent with different mitochondrial preparations, these parameters were routinely determined with each preparation. Great care was

Table 2. Effects of external Mg^{2+}, adenine nucleotides and NaCl on changes in $[Mg^{2+}]_m$

Values of $[Mg^{2+}]_m$ (mM) were determined in medium B (see the Experimental section) by using the fluorescence-ratio method (see the text). *mag-fura-2 loaded; mag-indo-1 loaded. Dashes indicate values not determined.

Substrate	Time (min)	$[Mg^{2+}]_m$ (mM)							
		External $[Mg^{2+}]$ (mM)							
		0.0	0.6	2.0	4.0	6.0	0.5 mM-ADP	0.5 mM-ATP	10 mM-NaCl
5 mM-succinate*	0.5	1.2	–	1.7	–	2.0	1.6	1.5	1.5
	5.0	0.8	–	1.7	–	2.4	0.60	0.7	1.2
5 mM-pyruvate† plus 1 mM-malate	0.5	1.3	1.3	1.4	2.2	–	–	–	–
	5.0	0.6	0.8	0.9	2.1	–	–	–	–
5 mM-2-oxoglutarate† plus 1 mM-malonate	0.5	0.8	1.3	1.7	1.5	–	–	–	–
	5.0	0.4	0.7	1.2	1.4	–	–	–	–

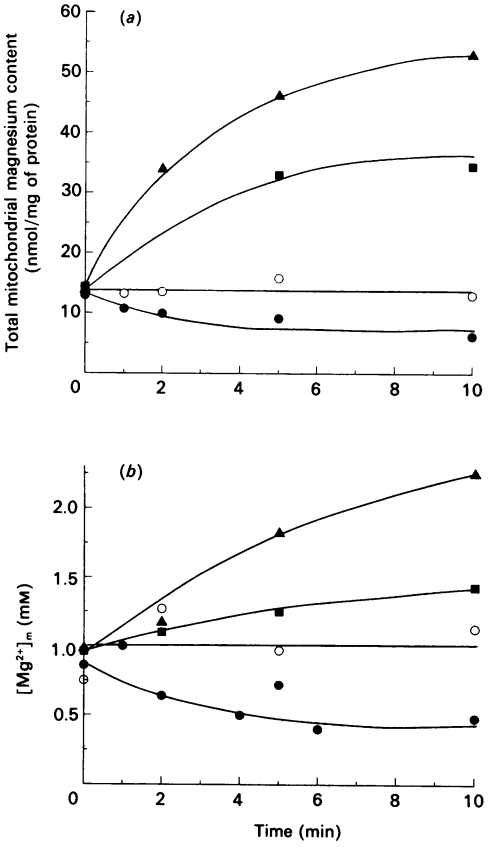

Fig. 5. Changes in (*a*) total magnesium content of dye-loaded mitochondria and (*b*) free matrix $[Mg^{2+}]$

(*a*) Mitochondria, loaded with either 100 μM-mag-fura-2 or 100 μM-mag-indo-1 (5 min, 30 °C), were incubated in medium A (see the Experimental section) with 1 μM-FCCP (○) or with 5 mM-Tris succinate plus no other additions (●), 2 mM-Mg^{2+} (■) or 15 mM-Mg^{2+} (▲). Samples (0.8 ml) were removed at the times indicated, and mitochondria sedimented (10000 *g*, 20 s), washed with 1 ml of ice-cold medium B, and re-sedimented as above. Total magnesium content was determined by atomic-absorption spectroscopy as detailed in the Experimental section. Values obtained in the presence of added Mg^{2+} were corrected for binding (< 5 nmol of Mg^{2+}/mg of protein) which was apparent at zero time. Data in the presence of succinate and zero $[Mg^{2+}]$ are the means of two experiments with mag-fura-2-loaded mitochondria, and one experiment with mag-indo-1-loaded mitochondria. Other measurements were made with mag-fura-2-loaded mitochondria (*b*). $[Mg^{2+}]_m$ was determined from fluorescence-ratio measurements which were performed in parallel with the total-magnesium measurements described in (*a*).

taken to correct for the contribution of non-Mg^{2+}-sensitive background fluorescence to the signals at each wavelength. This resulted from both substrate-induced (and time-dependent) changes in the mitochondrial NAD(P)H autofluorescence, and also non-Mn^{2+}-quenchable fluorescence of the remaining unhydrolysed dyes. This correction was made (1) by adding excess Mn^{2+} (in the presence of ionomycin plus FCCP) at the end of each run, or (2) by performing parallel incubations with unloaded (but preincubated) mitochondria. Results were essentially similar with either of these approaches. However, the latter approach allowed more precise measurement of time-dependent changes in NAD(P)H fluorescence, while neglecting the (constant) contribution from unhydrolysed dye.

A summary of the data obtained with mag-indo-1 and various substrates is given in Table 2. These data confirm that a time-dependent decline in $[Mg^{2+}]_m$ does occur upon addition of substrate in the absence of external Mg^{2+}. This decline was essentially unaffected by the presence of 0.5 mM-ADP, 0.5 mM-ATP or 10 mM-NaCl with 5 mM-succinate as substrate. As shown in Fig. 5, the fall in $[Mg^{2+}]_m$ was associated with a parallel decline in the total magnesium content of the mitochondria. Thus the decline in signal seems to represent a direct measure of the efflux of Mg^{2+} from the mitochondria. The observed initial rate of (total) Mg^{2+} efflux was approx. 3 nmol/min per mg of protein. This value is comparable with measurements by others [27–30].

Similarly, the apparent increase in $[Mg^{2+}]_m$ seen at increasing external Mg^{2+} concentrations by using the single-wavelength approach was also confirmed by using ratios (Fig. 5). As expected, this was matched by an increase in total mitochondrial Mg^{2+} content. The maximum rate of increase in total Mg^{2+} (at 15 mM external Mg^{2+}) in the presence of substrate was approx. 10 nmol/min per mg of protein, in agreement with previous measurements [27,29].

The immediate effect of substrate addition on $[Mg^{2+}]_m$ was investigated by using method (2) to correct for background fluorescence. Since addition of substrate is likely to cause changes in the mitochondrial concentration of Mg^{2+}-binding ligands, in particular ATP, it was expected that this might have a marked effect on $[Mg^{2+}]_m$. In fact, the observed changes in $[Mg^{2+}]_m$ were rather modest. For example, in the absence of external Mg^{2+}, and before the addition of substrate, $[Mg^{2+}]_m$ was measured at 1.4 ± 0.3 mM ($n = 3$) with mag-fura-2, and 1.5 ± 0.4 mM ($n = 3$) with mag-indo-1. Measured 30 s after the addition of 5 mM-succinate, these values were 1.1 ± 0.1 mM and 1.2 ± 0.2 mM respectively. These changes were associated with an increase in the mitochondrial ATP content, from below 0.05 nmol/mg to 1–2 nmol/mg.

DISCUSSION

Evaluation of the use of trapped fluorescent indicators to measure $[Mg^{2+}]_m$

Previous measurements [10,11] of intramitochondrial free Mg^{2+} concentration have relied on a 'null point' approach. This involves suspending de-energized mitochondria in a medium containing an impermeant Mg^{2+}-sensitive dye, and varying the ambient $[Mg^{2+}]$. The ambient Mg^{2+} concentration at which no change in signal occurs upon addition of bivalent-cation ionophore (A23187) is termed the 'null point'. The intramitochondrial $[Mg^{2+}]$ can then be calculated (see below) if the residual transmembrane pH gradient is known, since the transport of Mg^{2+} by A23187 involves exchange for $2H^+$ (see [10]).

The use of fluorescent indicators offers clear advantages over this approach. Firstly, the fluorescence approach is considerably more sensitive and straightforward, and does not require the determination of pH gradients, the precision of which is crucial to the estimate of $[Mg^{2+}]_m$ [10]. Secondly, the use of indicators trapped within the mitochondrial matrix allows $[Mg^{2+}]_m$ to be monitored continuously in fully energized respiring (or non-respiring) mitochondria. Of the two indicators used, we have found mag-indo-1 to be the indicator most suitable for these measurements, displaying the greatest shift in fluorescence intensity with changing $[Mg^{2+}]$ and least interference from background fluorescence.

Like fura-2 and indo-1, the use of the fluorescent Mg^{2+} indicators may, however, be limited to mitochondria from certain tissues only, owing to leakage of the hydrolysed dyes. For example, preliminary experiments have indicated that leakage of both Mg^{2+} indicators occurs rapidly from rat liver mitochondria, whereas mag-indo-1, but not mag-fura-2, is largely retained within mitochondria from white adipose tissue (G. A. Rutter, N. J. Osbaldeston, J. G. McCormack & R. M. Denton, unpublished work; see also [19]). Continuous perifusion of immobilized mitochondria [19] may provide one solution to this leakage problem.

The values of $[Mg^{2+}]_m$ obtained when using the fluorescent dyes rely critically on the apparent K_d values for Mg^{2+} within the mitochondrial matrix. In these studies we have attempted to obtain K_d values by using bivalent-metal ionophores in the presence of uncoupler. The values obtained, however, were 1.5–3.0-fold higher than those of the free acid forms of the dyes. This discrepancy would not seem to be due to the incomplete hydrolysis of the dye esters, since values close to those for the free dyes were obtained for the dyes generated within the mitochondrial matrix after complete mitochondrial disruption with Triton (see the Results section). Rather, it would appear that some property of the mitochondrial matrix environment (e.g. ionic strength or viscosity) influences the K_d values of the dyes, as reported for fura-2 [31]. Based on the above K_d values, measurements with either mag-indo-1 or mag-fura-2-loaded mitochondria gave values of close to 1 mM for $[Mg^{2+}]_m$, before, or immediately after, substrate addition. This estimate is comparable with, though slightly higher than, previous measurements based on the 'null point' approach. Thus Corkey *et al.* [10] obtained a value of 0.38 mM for $[Mg^{2+}]_m$ with liver mitochondria (at a total magnesium content of 40 nmol/mg). With bovine heart mitochondria, Jung & Brierley [11] obtained a value of 0.2 mM at a total magnesium content of 15 nmol/mg, typical for rat heart mitochondria (see the Results section and also [28]). This latter estimate for $[Mg^{2+}]_m$ within bovine heart mitochondria may, however, be an underestimate of 2–4-fold, since the authors did not correct for a residual pH gradient (0.1–0.2 pH unit, acid interior) in the acetate medium used. Hence a value for $[Mg^{2+}]_m$ of between 0.4 and 0.8 mM may represent a more accurate estimate from these experiments.

Since no changes in the total mitochondrial magnesium content were detected in the present studies, either during lengthy storage at 0 °C or during the loading period (see the Results section), it would seem reasonable to extrapolate to a value of about 1 mM for $[Mg^{2+}]_m$ for the situation *in vivo*. This implies that only a small gradient of Mg^{2+}, perhaps near unity, exists across the mitochondrial inner membrane, given recent measurements with mag-fura-2 of cytosolic $[Mg^{2+}]$ within isolated chick heart (0.5 mM; [13]) and rat liver (0.6 mM; [12]) cells. Further, recent measurements using a highly selective Mg^{2+} electrode [32] have given a value of 0.85 mM for cytosolic $[Mg^{2+}]$ within ferret ventricular cells.

A value for mitochondrial free $[Mg^{2+}]$ *in vivo* has been obtained from measurements of [isocitrate]/[citrate] ratio (assuming equilibrium at the aconitase reaction), by Tischler *et al.* [33]. These authors estimated a value of 2.7 mM for $[Mg^{2+}]_m$ within mitochondria rapidly isolated from hepatocytes. This approach gave a value of 0.8 mM for cytosolic $[Mg^{2+}]$ in these cells.

Nature of the Mg^{2+}-binding sites within mitochondria

Our results imply that, over the range studied, approx. 5–10% of total mitochondrial Mg^{2+} is free (assuming a matrix volume of 1 μl/mg of protein [2]), and that $[Mg^{2+}]_m$ is buffered against changes in the concentration of Mg^{2+}-binding species such as ATP. It follows that chelation of Mg^{2+} stemming from increases in intramitochondrial ATP concentration would seem an unlikely means of controlling the activity of mitochondrial Mg^{2+}-sensitive enzymes, contrary to certain models of the control of mitochondrial substrate oxidation [34].

The nature and concentration of the ligand(s) responsible for binding Mg^{2+} within heart mitochondria is not clear. Significantly, the proportions of total and free magnesium differ greatly from those of intramitochondrial calcium (< 0.1% free) [1,14–19]. This implies the existence of intramitochondrial molecules with great selectivity for the binding of Ca^{2+} over that of Mg^{2+}, as observed with permeabilized liver mitochondria [10].

Recent studies [13] on isolated heart cells loaded with mag-fura-2 have suggested that changes in intracellular $[Ca^{2+}]$ may cause increases in cytosolic $[Mg^{2+}]$, perhaps through competition for common (Ca^{2+}/Mg^{2+}) binding sites. We have attempted to investigate whether a similar inter-relationship exists within mitochondria, but without success. This was due to the sensitivity of both Mg^{2+} indicators to > 1 μM-Ca^{2+} (K_d close to 10 μM; see Table 1), a problem compounded by the likelihood of gradients of Ca^{2+} across the mitochondrial inner membrane [1,15–17]. Thus investigation of the effects of changes in mitochondrial $[Ca^{2+}]$ on $[Mg^{2+}]_m$ would appear to await the availability of Mg^{2+} indicators with greatly improved selectivity for Ca^{2+} over Mg^{2+}. Whether the rather poor discrimination between Ca^{2+} and Mg^{2+} of mag-fura-2 represents a potential problem in interpreting the data of Murphy *et al.* [13] perhaps requires clarification.

Uptake and efflux of Mg^{2+} by heart mitochondria

The use of the Mg^{2+}-sensitive indicators mag-indo-1 and mag-fura-2 provides a convenient means of studying the transport of Mg^{2+} across the mitochondrial inner membrane. Thus changes in free $[Mg^{2+}]$ occurred which paralleled changes in total mitochondrial magnesium content. In particular, efflux of Mg^{2+} was confirmed as an energy-dependent process requiring an oxidizable substrate and being blocked by uncoupler [27]. The observations that valinomycin enhanced, but nigericin blocked (Fig. 4), the efflux, indicate a requirement for the process of a significant pH gradient, but not high membrane potential. These observations

Table 3. Published $K_{0.5}$ values for the activation of PDHP phosphatase by Mg^{2+}, with various preparations derived from heart and adipose-tissue mitochondria

Values are at pH 7.2 and 30 °C and are taken from the references given in square brackets, from which full details can be obtained. Values were obtained either in the presence of EGTA (no Ca^{2+}) or in the presence of a saturating concentration of Ca^{2+} (25–100 μM). Results are from rat heart or rat epididymal adipose tissue, except those indicated by *, which were obtained with enzyme derived from pig heart. For A23187-permeabilized mitochondria and toluene-permeabilized mitochondria [procedure (i)], $K_{0.5}$ values were calculated from changes in the steady-state PDH activity in the presence of MgATP, so that PDH kinase is active. For the toluene-permeabilized mitochondria [procedure (ii)], $K_{0.5}$ values were calculated from the increase in PDH activity after 5 min in the absence of MgATP (and hence of PDH kinase). Dashes indicate values not available.

	$K_{0.5}$ value for Mg^{2+} (mM)			
	Heart		Adipose tissue	
	No Ca^{2+}	Ca^{2+}	No Ca^{2+}	Ca^{2+}
Isolated PDHP phosphatase	3.1*	1.1* [5]	3.2	1.4 [5]
A23187-permeabilized mitochondria	–	–	2.6	1.1 [5]
Toluene-permeabilized mitochondria				
Procedure (i)	0.07	0.03 [7]	0.33	0.20 [6]
Procedure (ii)	0.13	0.08 [36]	0.60	0.32 [6]

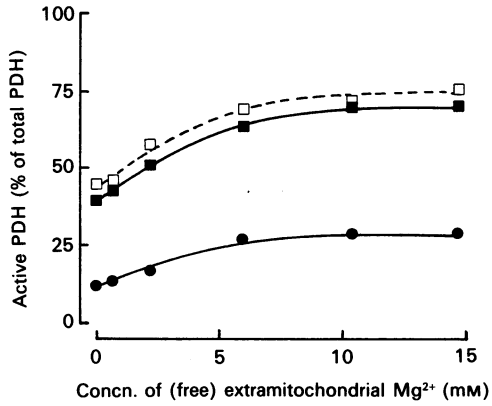

Fig. 6. Effects of changes in extramitochondrial Mg^{2+} concentration on the amount of active PDH within intact, fully coupled, rat heart mitochondria

Fresh (●, ■) or mag-fura-2-loaded (as in Fig. 2; □) mitochondria (approx. 0.5 mg of protein/ml) were incubated at 30 °C in 1 ml of medium A (see the Experimental section) containing 10 mM-2-oxoglutarate and 0.25 mM-L-malate either with (□, ■) or without (●) 0.25 mM-pyruvate. Sufficient $MgCl_2$ was added to give the required free $[Mg^{2+}]$, 8 min before the subsequent determination of the level of active PDH (see ref. [38] for details of the extraction and assay of PDH); total PDH was unaffected by the treatments. Essentially similar results were obtained after 4 min incubations, or after incubation for 4 min after a 4 min pre-incubation in the absence of added Mg^{2+} (not shown for clarity). Results shown are the means of measurements made on at least 3 separate mitochondrial preparations; S.E.M. values were all within 15% of the values shown.

have also been made in earlier studies of total magnesium movements [28–30,35].

Significance of the value of $[Mg^{2+}]_m$ to the regulation of intramitochondrial enzymes by Ca^{2+}

We have recently reported [4,20] that the sensitivity of NAD^+–isocitrate dehydrogenases to Ca^{2+} ($K_{0.5}$ value 5–43 μM) may be markedly less than that of the PDH system [7] or 2-oxoglutarate dehydrogenase at 1 mM-Mg^{2+} ($K_{0.5}$ value for Ca^{2+} below 1 μM). Indeed, only at Mg^{2+} concentrations below about 0.1 mM did the $K_{0.5}$ values for Ca^{2+} of NAD^+–isocitrate dehydrogenase approach those of latter enzymes. Similarly, the $K_{0.5}$ value for the inhibition of mitochondrial pyrophosphatase by Ca^{2+} at 1 mM-Mg^{2+} appears to be about 10 μM [3], falling to sub-micromolar values only at Mg^{2+} concentrations below 0.1 mM. Thus, if the value for intramitochondrial $[Mg^{2+}]$ of about 1 mM pertains *in vivo*, then these differences in the Ca^{2+} sensitivities may extend the range of Ca^{2+} concentrations to which mitochondrial oxidative metabolism is sensitive.

Table 3 summarizes some recent estimates of the $K_{0.5}$ values for Mg^{2+} of the activation of heart and adipose-tissue PDHP phosphatase in various preparations. In all cases, the presence of Ca^{2+} results in a marked decrease in the $K_{0.5}$ value. With the isolated enzyme, a substantial increase in $V_{max.}$ is also apparent, whereas no change in $V_{max.}$ is observed in the preparations of mitochondria permeabilized with either A23187 or toluene. This is presumably because the concentration of PDHP phosphatase used in the assays with the extracted enzyme is very much lower than that within the permeabilized mitochondria (see [6]). The values of $K_{0.5}$ for Mg^{2+} obtained with toluene-permeabilized mitochondria are considerably lower than the corresponding values obtained with either A23187-permeabilized mitochondria or the isolated phosphatase preparations. On the basis of the use of fluorescent indicators, the concentration of Mg^{2+} found in freshly prepared intact coupled mitochondria from heart (the present work) and rat epididymal adipose tissue (G. A. Rutter, unpublished work) is about 1 mM. In both cases, addition of Ca^{2+} to intact coupled mitochondria causes a 4–6-fold increase in PDH activity [21,37]. Assuming that Ca^{2+} has no effect on the $V_{max.}$ of the phosphatase within the mitochondria, then these observations imply that the $K_{0.5}$ values for Mg^{2+} within intact mitochondria must lie in the mM range. This view is supported by the data presented in Fig. 6. This demonstrates that, in the absence of Ca^{2+}, changes in the extramitochondrial Mg^{2+} concentration (and hence $[Mg^{2+}]_m$; see Figs. 4 and 5) over the ranges used in the present paper can lead to changes in the amount of active PDH within the matrix. Again, this emphasizes the importance of being able to measure $[Mg^{2+}]_m$.

The mechanism underlying the apparent sensitization of the PDH system to Mg^{2+} in toluene-permeabilized mitochondria remains to be establish. Possible explanations include alterations in the interaction of the PDH complex with the mitochondrial inner membrane or the loss of some low-molecular-mass regulator of the phosphatase as a result of the permeabilization with toluene.

Since the submission of this article, a paper has appeared by Jung *et al.* [39], using a similar approach to that of the present work, but using mag-fura-2-loaded bovine heart mitochondria. These authors reported rather lower matrix Mg^{2+} concentrations (around 0.5 mM). However, this discrepancy can easily be explained, as these authors did not allow for any changes in K_d when the dye is located within the mitochondrial matrix (Table 1).

This work was supported by the Medical Research Council (U.K.) and the Lister Institute. J.G.McC. is a Lister Institute Research Fellow. We thank Dr. Stuart Telfer (Department of Animal Physiology and

Nutrition, University of Leeds) for performing atomic-absorption measurements, and Dr. A. P. Halestrap (University of Bristol) and Dr. R. G. Hansford (National Institutes of Health, Baltimore, MD, U.S.A.) for useful discussion.

REFERENCES

1. McCormack, J. G., Halestrap, A. P. & Denton, R. M. (1990) Physiol. Rev. **70**, 391–425
2. Halestrap, A. P. (1989) Biochim. Biophys. Acta **973**, 355–382
3. Davidson, A. M. & Halestrap, A. P. (1989) Biochem. J. **258**, 817–821
4. Rutter, G. A. & Denton, R. M. (1989) Biochem. J. **263**, 445–452
5. Thomas, A. P., Diggle, T. A. & Denton, R. M. (1986) Biochem. J. **238**, 83–91
6. Midgley, P. J. W., Rutter, G. A., Thomas, A. P. & Denton, R. M. (1987) Biochem. J. **241**, 371–377
7. Rutter, G. A., Midgley, P. J. W. & Denton, R. M. (1989) Biochim. Biophys. Acta **1014**, 263–270
8. McCormack, J. G. & Denton, R. M. (1979) Biochem. J. **180**, 533–544
9. Thomas, A. P. & Denton, R. M. (1986) Biochem. J. **238**, 93–101
10. Corkey, B. A., Duszynski, J., Rich, T. L., Matchinsky, B. & Williamson, J. R. (1976) J. Biol. Chem. **261**, 2567–2574
11. Jung, D. W. & Brierley, G. P. (1986) J. Biol. Chem. **261**, 6408–6414
12. Raju, B., Murphy, E., Levy, L. A., Hall, R. D. & London, R. E. (1989) Am. J. Physiol. **256**, C540–C548
13. Murphy, E., Freudenrich, C. C., Levy, L. A., London, R. E. & Lieberman, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2981–2984
14. Lukacs, G. L. & Kapus, A. (1987) Biochem. J. **248**, 609–613
15. McCormack, J. G., Browne, H. M. & Dawes, N. J. (1989) Biochim. Biophys. Acta **973**, 420–427
16. Moreno-Sanchez, R. & Hansford, R. G. (1988) Biochem. J. **256**, 403–412
17. Wan, B., LaNoue, K. F., Cheung, J. Y. & Scaduta, R. C., Jr. (1989) J. Biol. Chem. **264**, 13430–13439
18. Davis, M. H., Altshuld, R. A., Jung, D. W. & Brierley, G. P. (1987) Biochem. Biophys. Res. Commun. **149**, 40–45
19. Reers, M., Kelly, R. A. & Smith, T. W. (1989) Biochem. J. **257**, 131–142
20. Rutter, G. A. & Denton, R. M. (1988) Biochem. J. **252**, 181–189
21. Denton, R. M., McCormack, J. G. & Edgell, N. E. (1980) Biochem. J. **190**, 107–117
22. Stanley, P. E. & Williams, S. G. (1969) Anal. Biochem. **29**, 381–392
23. Gornall, H. G., Bardawill, C. J. & David, M. N. (1949) J. Biol. Chem. **177**, 751–756
24. Tsien, R. Y. & Poenie, M. (1986) Trends Biochem. Sci. **11**, 450–455
25. Cobbold, P. H. & Rink, T. J. (1988) Biochem. J. **248**, 313–328
26. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
27. Crompton, M., Capano, M. & Carafoli, E. (1976) Biochem. J. **154**, 735–742
28. Akerman, K. E. O. (1981) J. Bioenerg. Biomembr. **13**, 133–139
29. Brierley, G. P., Davis, M. & Jung, D. W. (1987) Arch. Biochem. Biophys. **253**, 322–332
30. Brierley, G. P., Davis, M. H. & Jung, D. W. (1988) J. Bioenerg. Biomembr. **20**, 229–242
31. Williams, D. A. & Fay, F. S. (1990) Cell Calcium **11**, 75–83
32. Buri, A., & MacGuigan, J. A. (1990) Exp. Physiol., in the press
33. Tischler, M. E., Friedrichs, D., Coll, K. & Williamson, J. R. (1977) Arch. Biochem. Biophys. **184**, 222–236
34. Garfinkel, D., Kohn, M. C. & Achs, M. J. (1979) Am. J. Physiol. **237**, R181–R186
35. Diwan, J. J. (1987) Biochim. Biophys. Acta **895**, 155–165
36. Rutter, G. A. (1988) Ph.D. Thesis, University of Bristol
37. Marshall, S. E., McCormack, J. G. & Denton, R. M. (1984) Biochem. J. **214**, 249–260
38. McCormack, J. G. & Denton, R. M. (1989) Methods Enzymol. **174**, 95–118
39. Jung, D. W., Apel, A. & Brierley, G. P. (1990) Biochemistry **29**, 4121–4128

Received 11 May 1990/18 June 1990; accepted 9 July 1990