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Measurement of the matrix free Ca^{2+} concentration in heart mitochondria by entrapped fura-2 and quin2

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A method was developed to monitor continuously the matrix free Ca^{2+} concentration ($[Ca^{2+}]_m$) of heart mitochondria by use of the fluorescent Ca^{2+} indicators, fura-2 and quin2. The acetoxymethyl esters of fura-2 and quin2 were accumulated in and hydrolysed by isolated mitochondria. An increase of the mitochondrial Ca content from 0.3 nmol/mg of protein to 6 nmol/mg corresponded to a rise of $[Ca^{2+}]_m$ from 30 to 1000 nM. The results indicate that physiological fluctuations of the mitochondrial Ca content elicit changes of $[Ca^{2+}]_m$ in that range which regulates the matrix dehydrogenases.

INTRODUCTION

Isolated mitochondria have a large capacity to accumulate Ca^{2+} and buffer the extramitochondrial $[Ca^{2+}]$ at around 1 μM if their Ca content exceeds 10 nmol/mg of protein (reviewed by Åkerman & Nicholls, 1983; Crompton, 1985; Hansford, 1985). In contrast with this, it was revealed that in resting cells the cytoplasmic $[Ca^{2+}]$ was around 100 nM (Tsien *et al.*, 1982; Williams *et al.*, 1985) and the mitochondria *in situ* contained 0.5–2 nmol/mg of protein (Wendt-Gallitelli & Jacob, 1982; Crompton *et al.*, 1983; Somlyo *et al.*, 1985). Therefore the role of the mitochondria as the main intracellular Ca^{2+} buffer became unlikely (Crompton, 1985; Hansford, 1985). According to a later theory, the Ca^{2+}-transport pathways relay the changes of the cytoplasmic $[Ca^{2+}]$ to the mitochondrial matrix space, where the activities of three dehydrogenases (pyruvate dehydrogenase, NAD^+–isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase) are regulated by $[Ca^{2+}]_m$ (Denton & McCormack, 1980, 1985). This theory is valid only if the value of $[Ca^{2+}]_m$ is in the range of 0.05–5 μM, where the intramitochondrial dehydrogenases are regulated by Ca^{2+} (for reviews see Denton & McCormack, 1980, 1985; Hansford, 1985). So far the only approach to estimate $[Ca^{2+}]_m$ was the 'null-point titration technique' (Hansford & Castro, 1982; Coll *et al.*, 1982), which required permeabilization of mitochondria by the Ca^{2+} ionophore A23187 (which transports Mg^{2+} as well as Ca^{2+}), measurements of Ca^{2+} fluxes and ΔpH at various extramitochondrial $[Ca^{2+}]$. The use of Ca^{2+}-sensitive fluorescent indicators (quin2 and fura-2) reported in the present paper offers the advantages of higher Ca^{2+}-sensitivity, and the possibility of continuous and direct monitoring of $[Ca^{2+}]_m$ independently of the ΔpH in the absence of Mg^{2+} flux. Some of the results were presented in a preliminary form (Lukács *et al.*, 1987).

MATERIALS AND METHODS

Ionomycin, fura-2/AM, fura-2, quin2/AM, quin2 and CCCP were purchased from Calbiochem. Ruthenium Red from BDH was used after recrystallization as described by Luft (1971).

Rat heart mitochondria were prepared as described by Lukács & Fonyó (1986), except that the washing and the final resuspension medium contained 240 mM-sucrose, 5 mM-Tris/HCl, 0.1% (w/v) bovine serum albumin and 0.6 mM-EGTA at pH 7.4. EGTA prevented any accumulation of Ca^{2+} during the preparation procedure.

Fluorescence of the mitochondrial suspension was monitored with a Jobin Yvon JY3 spectrofluorimeter at 22–24 °C. The excitation wavelength was 339 nm (4 nm slit) and the emission wavelength was either 493 nm or 500 nm (4 nm slits), for quin2- or fura-2-loaded mitochondria respectively. Triton X-100 addition did not change the Ca^{2+}-sensitivity of the free acid form of the dyes monitored in the presence of control mitochondria.

To load mitochondria with fluorescent Ca^{2+} indicators, mitochondria (approx. 50 mg of protein/ml) were incubated for 30 min at 35 °C in the presence of 300 μM-quin2/AM or 6 μM-fura-2/AM. A 20-fold dilution was then carried out with the final suspension medium (4 °C), and mitochondria were re-centrifuged to obtain the stock suspension (40–50 mg of protein/ml). No quin2 and fura-2 were taken up in the matrix space if mitochondria were incubated with them instead of their acetoxymethyl ester forms. There was no difference in extent of Ca^{2+} uptake, generation of membrane potential (170 mV, measured by a tetraphenylphosphonium-sensitive electrode as in Ligeti & Lukács, 1984) and respiratory control between dye-loaded and control mitochondria (results not shown).

The matrix free Ca^{2+} concentrations were calculated using the formula $[Ca^{2+}]_m = K_d(F-F_{min.})/(F_{max.}-F)$ and $F_{min.} = F_o + kF_{max.}$, where K_d is the dissociation constant for Ca^{2+} binding to the indicator dye, F is arbitrary fluorescence unit, F_o is the autofluorescence and the correction factor, k, for quin2 and fura-2 is 0.17 and 0.12 respectively (see Hesketh *et al.*, 1983; Pollock *et al.*, 1986). In the presence of succinate, the steady-state fluorescence signal was not further elevated by Ca^{2+} (1 mM) plus ionomycin (4 μM; results not shown); thus it represented $F_{max.}$. The value of $F_{max.}$ might be under-

Abbreviations used: CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $[Ca^{2+}]_m$, intramitochondrial (matrix) free $[Ca^{2+}]$; quin2/AM and fura-2/AM, acetoxymethyl esters of quin2 and fura-2; ΔpH, pH difference across the mitochondrial inner membrane.

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estimated if the matrix space contained heavy-metal ions, which are able to quench the fluorescence of the Ca–dye complex. Addition of the membrane-permeant heavy-metal chelator TPEN [100 μM-*NNN'N'*-tetrakis-(2-pyridylmethyl)ethylenediamine (Arslan *et al.*, 1985)] did not enhance the $F_{max.}$, excluding its underestimation. For determination of F_0, Mn^{2+} (1.5 mM) was added before mitochondria. The fluorescence obtained was not further decreased by repetitive additions of Mn^{2+} (1.5 mM) plus ionomycin (2 μM). For quin2 and fura-2 the K_d values were taken to be 60 nM and 135 nM respectively (Grynkiewicz *et al.*, 1985), as the matrix space contained approx. 10 nmol of Mg^{2+}/mg of protein, determined by atomic-absorption photometry (corresponding to approx. 0.1 mM matrix free Mg^{2+}; see Corkey *et al.*, 1986). The matrix pH must have been above 7.4, since the respiring mitochondria were suspended in the basic medium (pH 7.4 at 22–24 °C). The Ca^{2+}-binding properties of the dyes are independent of the pH above 7.0 (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985). The dye leakage was tested by Mn^{2+} addition in the presence of Ruthenium Red (see Fig. 2*f*); it never exceeded 5% of ($F_{max.} - F_0$) by the end of the experiments.

Ca^{2+} uptake was monitored by a Ca^{2+}-sensitive electrode and calibrated in the presence of Ruthenium Red (Spät *et al.*, 1987). Uptake was corrected for adsorption in the presence of Ruthenium Red (13 μM) plus CCCP (2 μM).

The initial Ca (and Mg) contents of dye-loaded mitochondria were determined after centrifugation (12000 *g*, 2 min) of 4–5 mg of mitochondrial protein in 120 mM-KCl/20 mM-Tris/Hepes/0.4 mM-EGTA at 0 °C. The pellet was digested with 0.16 ml of conc. HCl and 0.04 ml of conc. HNO_3 for 3 h at 70 °C and supplemented with 0.9 ml of 0.22% $LaCl_3$ and 0.11% CsCl solution. Ca^{2+} and Mg^{2+} concentrations were determined with a Pye–Unicam atomic-absorption spectrometer, with an air/acetylene flame.

RESULTS AND DISCUSSION

Quin2 and fura-2 added as their acetoxymethyl esters (quin2/AM and fura-2/AM) have to be hydrolysed to gain Ca^{2+}-sensitivity and to be retained in any membrane-enclosed space (Tsien, 1981; Grynkiewicz *et al.*, 1985). To assess the question of whether the isolated mitochondria possess hydrolytic activity, we tested the appearance of Ca^{2+}-dependent and Mn^{2+}-sensitive fluorescence (Hesketh *et al.*, 1983; Grynkiewicz *et al.*, 1985) in the mitochondrial suspension (Mn^{2+} does not quench the fluorescence of the acetoxymethyl ester dyes; see Schackman & Chock, 1986). Data reported in Fig. 1 show that both dyes were hydrolysed by unidentified esterase(s), and this process was essentially complete within 30 min.

For further experiments, the loaded mitochondrial suspension was diluted and re-centrifuged to wash out the extramitochondrial dye.

The following findings show that de-esterification occurred in a vesicular space, and the free dyes were retained there. (1) No significant Mn^{2+}- or Ca^{2+}-sensitive fluorescence was observed in the supernatant obtained after separation of the loaded mitochondria from the incubation medium (Fig. 2*a*). (2) At the same time, considerable Mn^{2+} quenching was detected in the suspension of mitochondria (Figs. 2*b* and 2*e*). (3) Decreasing the extramitochondrial Ca^{2+} to 5 nM by the addition of EGTA and Tris base (to raise the pH of the medium to 8.3) did not alter the fluorescence signal (Fig. 2*c*). (4) EGTA decreased the fluorescent signal only in the presence of the Ca^{2+} ionophore ionomycin (Figs. 2*c* and 2*d*), which released intramitochondrial Ca^{2+}.

To verify that the dye was entrapped in the mitochondrial matrix space and not in any putative contaminating subcellular organelle, we took advantage of the specific properties of mitochondrial Ca^{2+} transport. Energization of the mitochondria by a respiratory substrate, succinate, caused a rapid Ca^{2+} uptake via the uniporter (as measured by the Ca^{2+}-sensitive electrode) and at the same time an increase in the fluorescence signal (Figs. 2*e* and 3). Mn^{2+} added subsequently quenched the fluorescence signal, and the quench was more rapid in the presence of succinate than in its absence (Fig. 2*e*, cf. Fig. 2*b*), owing to the enhanced Mn^{2+} uptake through the uniporter, driven by the high membrane potential (Puskin *et al.*, 1976). In our experiments Mn^{2+} uptake and its inhibition by Ruth-

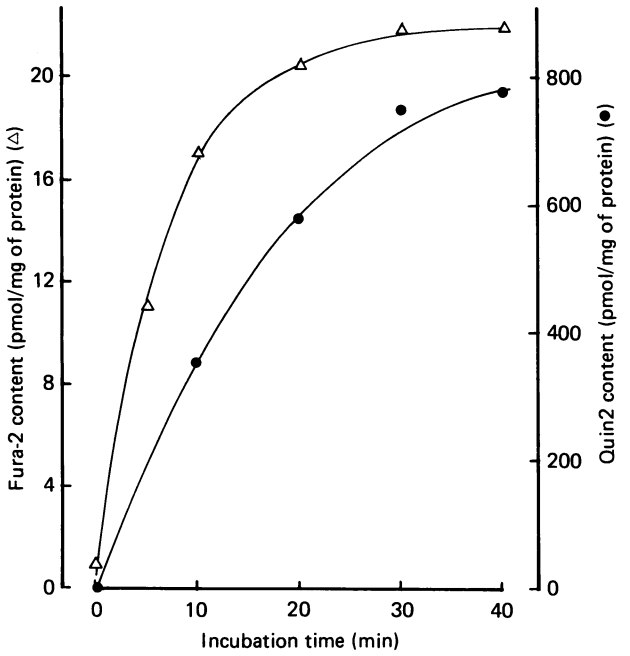

Fig. 1. Appearance of the Mn^{2+}-sensitive fluorescence signal of quin2 and fura-2 in the suspension of isolated heart mitochondria

Mitochondria (50 mg of protein/ml) were incubated with a final concentration of 300 μM-quin2/AM (●) or 6 μM-fura-2/AM (△). At the times indicated, samples equivalent to 1 mg of mitochondrial protein were transferred to the measuring cuvette, containing 2 ml of the basic medium (240 mM-sucrose, 20 mM-Tris/HCl, 2 mM-KCl, 200 μM-Ca^{2+}, 3 μM-rotenone, 0.1% albumin, pH 7.4) supplemented with 0.063% Triton X-100. The fluorescence of the Ca^{2+}-saturated dye was quenched by 1.5 mM-$MnCl_2$. The amount of hydrolysed dyes was determined by comparing the fluorescence of the incubated and lysed mitochondria with the fluorescence of known amounts of quin2 and fura-2 free acid in the presence of lysed unloaded mitochondria and expressed as pmol/mg of mitochondrial protein. No Mn^{2+} quench or Ca^{2+}-sensitive fluorescence was observed in control mitochondria, preincubated only with 0.75% (v/v) dimethyl sulphoxide, the solvent of the dyes.

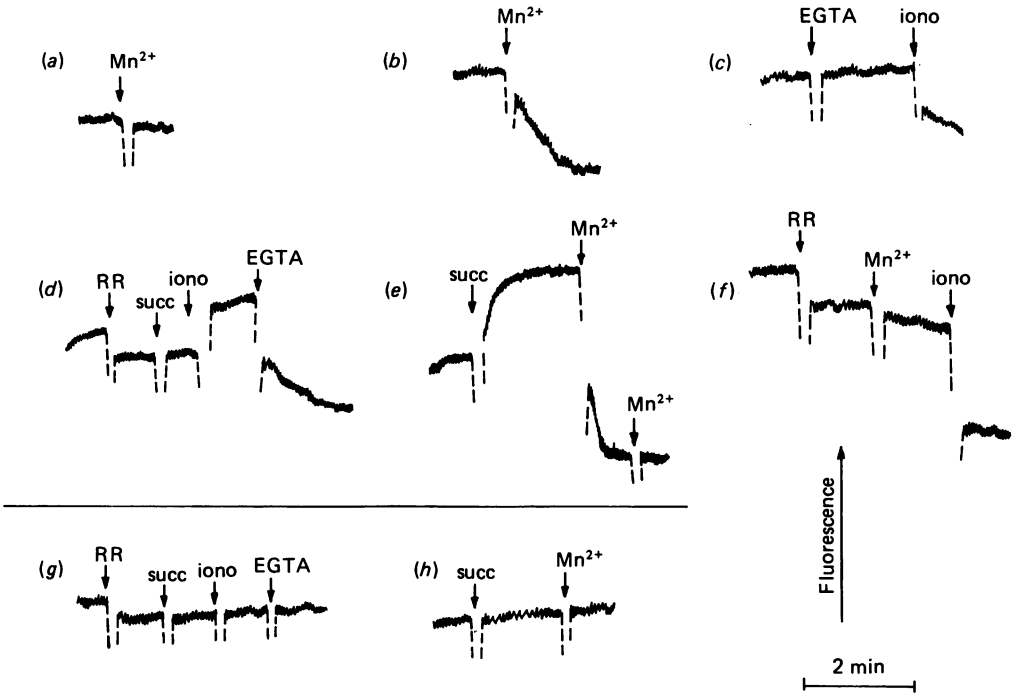

Fig. 2. Fluorescence changes of quin2, indicating the intramitochondrial localization of the dye

Quin2-loaded mitochondria (0.4 mg of protein/ml) were incubated in the basic medium. In (*a*) 2 ml of the suspension was centrifuged immediately (12000 *g*, 2 min) and fluorescence was measured in the supernatant. In (*g*) and (*h*) control mitochondria (0.4 mg/ml, preincubated only with dimethyl sulphoxide) were used. Further additions: 1.5 mM-$MnCl_2$ (Mn^{2+}), 5 mM-Tris/succinate (succ), 13 μM-Ruthenium Red (RR), 2 μM-ionomycin (iono), 3 mM-EGTA with 20 mM-Tris base (EGTA). Records are from one of eight similar experiments.

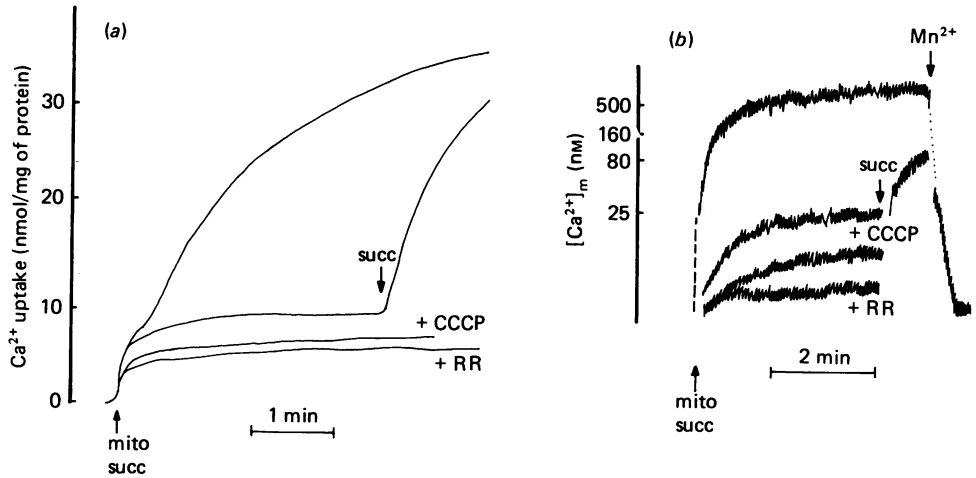

Fig. 3. Measurement of the Ca^{2+} uptake (*a*) and the concomitant change in $[Ca^{2+}]_m$ (*b*) of quin2-loaded mitochondria

Quin2-loaded mitochondria (mito; 0.4 mg of protein/ml) were added to the basic medium containing 5 mM-Tris/succinate (succ), except when Tris/succinate was added later as indicated on the Figure. Fluorescence was measured as in Fig. 1. Ruthenium Red (RR; 13 μM) or CCCP (2 μM) was added before mitochondria, and 1.5 mM-$MnCl_2$ (Mn^{2+}) when indicated. The quenching effect of Ruthenium Red was not corrected.

enium Red were verified indirectly by the changes of membrane potential (results not shown).

Ruthenium Red, a specific inhibitor of the uniporter-mediated cation (Ca^{2+} or Mn^{2+}) uptake (Moore, 1971), diminished both the succinate-induced rise in fluorescence (Fig. 2*d*) and the quenching effect of Mn^{2+} (Fig. 2*f*). Subsequently added ionomycin, which transports both Ca^{2+} and Mn^{2+}, increased the fluorescence in the presence of Ca^{2+} (Fig. 2*d*) but decreased it when Mn^{2+} (1.5 mM) was also present (Fig. 2*f*). The same results were obtained with fura-2-loaded mitochondria. The autofluorescence of mitochondria preincubated with dimethyl sulphoxide instead of fluorescent indicators was influenced only by Ruthenium Red (Figs. 2*g* and 2*h*). All

these observations confirmed the intramitochondrial localization of quin2 and fura-2.

Fig. 3 shows the parallel measurements of Ca^{2+} uptake by Ca^{2+}-sensitive electrode and $[Ca^{2+}]_m$ with entrapped quin2 in the same mitochondrial preparation under conditions which interfere with Ca^{2+} uptake. Both the Ca^{2+} uptake and the concomitant increase in fluorescence were inhibited in a parallel manner by Ruthenium Red, by the uncoupler CCCP or by the lack of respiratory substrate (Fig. 3).

The relationship between mitochondrial Ca^{2+} content and $[Ca^{2+}]_m$ measured by two different indicators is illustrated in Fig. 4. The difference between the values of $[Ca^{2+}]_m$ obtained with the two indicators at the same Ca load probably originates from the higher Ca^{2+}-chelation capacity of quin2 (Ashley, 1986). From fura-2 37.0 ± 11.9 pmol/mg of protein ($\pm$S.E.M.; $n = 5$) was accumulated in the matrix space, whereas the amount of quin2 was 828 ± 106.8 pmol/mg ($n = 8$). Therefore $[Ca^{2+}]_m$ values obtained by fura-2 are much closer to those of mitochondria which are not loaded with indicator.

The mitochondrial Ca content *in situ* was found to be 0.5–2.0 nmol/mg of protein by electron-probe X-ray microanalysis (Wendt-Gallitelli & Jacob, 1982; Somlyo *et al.*, 1985). Similar results were obtained in isolated mitochondria both by the rapid fractionation technique (Reinhart *et al.*, 1984) and by conventional preparation provided that Ca^{2+} fluxes were inhibited during the isolation (Crompton *et al.*, 1983; Assimacopoulos-Jeannet *et al.*, 1986). In our case the freshly prepared mitochondria contained 0.95 ± 0.35 ($n = 5$) nmol of Ca/mg of protein, which decreased to 0.33 ± 0.16 nmol/mg during the dye-loading period. These Ca contents correspond to $[Ca^{2+}]_m$ values of about 100 nM and 30 nM respectively.

After hormonal stimulation, the Ca content of liver mitochondria increased to 6 nmol/mg of protein, and concomitantly the activities of two intramitochondrial dehydrogenases increased (Assimacopoulos-Jeannet *et al.*, 1986). In isolated heart mitochondria the relationship between the Ca content and dehydrogenase activities was similar (Hansford & Castro, 1982; Crompton *et al.*, 1983).

Our results show that in fura-2-loaded mitochondria a rise in the Ca content from 1 to 6 nmol/mg of protein corresponds to a change in $[Ca^{2+}]_m$ from 100 nM to 1000 nM. This $[Ca^{2+}]_m$ range is within the regulatory range of the intramitochondrial dehydrogenases (Denton & McCormack, 1985; Hansford, 1985). Thus the direct measurements of $[Ca^{2+}]_m$ strongly support the idea that physiological fluctuations of the mitochondrial Ca content are able to elicit appropriate changes in $[Ca^{2+}]_m$ to regulate the activity of the matrix dehydrogenases.

These experiments demonstrate that the fluorescent Ca^{2+} indicators can be hydrolysed and accumulated also in an isolated subcellular organelle. This may expand the field where these fluorescent Ca^{2+} indicators can be applied. On the other hand, the extent of accumulation of the dyes by mitochondria *in situ* should be considered.

While the manuscript was in preparation, we were informed that similar results were obtained in Gunter's laboratory in Rochester, NY, U.S.A. (T. E. Gunter, personal communication).

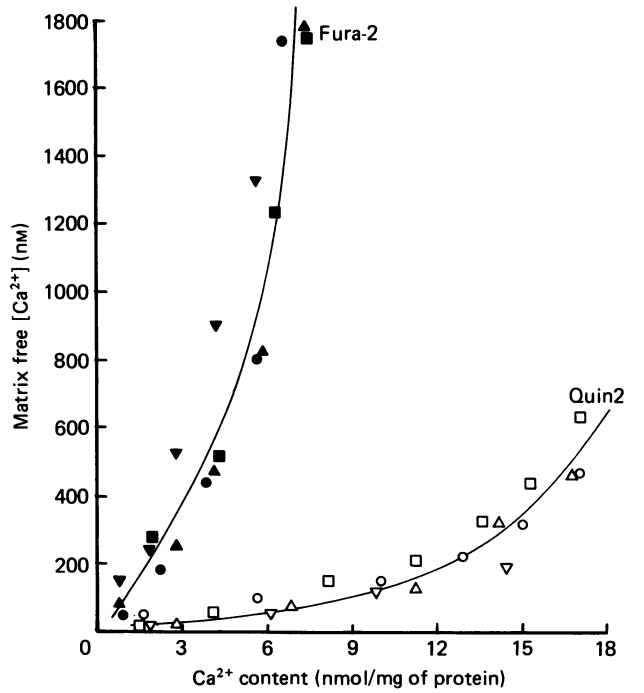

Fig. 4. Relationship between the mitochondrial Ca content and matrix free Ca^{2+} measured by fura-2 or quin2

Ca^{2+} uptake was initiated by addition of mitochondria loaded with fura-2 or quin2 (0.4 mg of protein/ml) to the basic medium supplemented with 5 mM-Tris/succinate. Ca^{2+} uptake and $[Ca^{2+}]_m$ were determined every 12 s. Ca content of the mitochondria was calculated as the sum of the initial Ca content, determined by atomic-absorption spectrometry, and of the accumulated Ca^{2+} monitored by the Ca^{2+}-selective electrode (as in Fig. 3). Data are from four separate experiments in which mitochondria were loaded with fura-2 (●, ▼, ▲, ■) or quin2 (○, ▽, △, □).

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