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Evidence for Two Compartments of Exchangeable Calcium in Isolated Rat Liver Mitochondria Obtained Using a ^{45}Ca Exchange Technique in the Presence of Magnesium, Phosphate, and ATP at 37° C

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Summary. The distribution of calcium between isolated rat liver mitochondria and the extramitochondrial medium at 37 °C and in the presence of 2 mM inorganic phosphate, 3 mM ATP, 0.05 or 1.1 mM free magnesium and a calcium buffer, nitrilotriacetic acid, was investigated using a ^{45}Ca exchange technique. The amounts of ^{40}Ca in the mitochondria and medium were allowed to reach equilibrium before initiation of the measurement of ^{45}Ca exchange. At 0.05 mM free magnesium and initial extramitochondrial free calcium concentrations of between 0.15 and 0.5 μM, the mitochondria accumulated calcium until the extramitochondrial free calcium concentration was reduced to 0.15 μM. Control experiments showed that the mitochondria were stable under the incubation conditions employed. The ^{45}Ca exchange data were found to be consistent with a system in which two compartments of exchangeable calcium are associated with the mitochondria. Changes in the concentration of inorganic phosphate did not significantly affect the ^{45}Ca exchange curves, whereas an increase in the concentration of free magnesium inhibited exchange. The maximum rate of calcium outflow from the mitochondria was estimated to be 1.7 nmol/min per mg of protein, and the value of $K_{0.5}$ for intramitochondrial exchangeable calcium to be about 1.6 nmol per mg of protein. Ruthenium Red decreased the fractional transfer rate for calcium inflow to the mitochondria while nupercaine affected principally the fractional transfer rates for the transfer of calcium between the two mitochondrial compartments. The use of the incubation conditions and ^{45}Ca exchange technique described in this report for studies of the effects of agents which may alter mitochondrial calcium uptake or release (e.g., the pre-treatment of cells with hormones) is briefly discussed.

Key words: Calcium transport, liver mitochondria, compartmental analysis, $^{45}Ca^{2+}$ exchange, magnesium and calcium transport

A number of hormones, neurotransmitters and cell stimuli, and cyclic AMP, have been shown to alter the distribution of intracellular calcium between the mitochondria and cytoplasm (Friedmann & Rasmussen, 1970; Babcock, Chen, Yip & Lardy, 1979; Blackmore, Dehaye & Exton, 1979; Borle & Uchikawa, 1979; Mikkelsen & Schmidt-Ullrich, 1980; Barritt, Parker & Wadsworth, 1981) or to induce changes in calcium inflow or outflow in mitochondria subsequently isolated from cells or tissues exposed to these agents (Yamazaki, 1975; Kimura & Rasmussen, 1977; Andia-Waltenbaugh, Kimura, Wood, Divakaran & Friedmann, 1978; Hughes & Barritt, 1978; Prpić, Spencer & Bygrave, 1978; Taylor, Prpić, Exton & Bygrave, 1980). In some cases strong evidence that the changes in calcium concentration are part of the mechanism by which agonists regulate metabolic reactions in the cytoplasm (Blackmore et al., 1979) and possibly mitochondria (Foldes & Barritt, 1977; Chan, Bacon & Hill, 1979; Denton, McCormack & Edgell, 1980) has been provided. The mechanisms by which agonists or cyclic AMP modify the quantity of calcium present in mitochondria are currently under investigation. Furthermore, consideration is now being given to the implications, with respect to the intracellular distribution of calcium, of the changes induced by hormones in the rates of calcium transport observed in isolated mitochondria (Czech, 1977; Barritt, 1980; Brand & De Selincourt, 1980). However, in most experiments so far conducted with isolated mitochondria, the incubation conditions chosen have differed appreciably from the physical and chemical environment of mitochondria in cells (Yamazaki, 1975; Kimura & Rasmussen, 1977; Andia-Waltenbaugh et al., 1978; Hughes & Barritt, 1978; Prpić et al., 1978; Taylor et al., 1980).

Since isolated mitochondria can rapidly take up large quantities of calcium from the suspension medium (reviewed by Lehninger, Carafoli & Rossi, 1967), the question of the cellular mechanisms which control

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the release of this accumulated calcium has also concerned many investigators (reviewed by Bygrave, 1978; Fiskum & Lehninger, 1980; Nicholls & Brand, 1980; and Lehninger, Vercesi & Bababunmi, 1978; Harris, Al-Shaikhaly & Baum, 1979; Lötscher, Winterhalter, Carafoli & Richter, 1979; Roman, Gmaj, Nowicka & Angielski, 1979; Haworth, Hunter & Berkoff, 1980; Juzu & Holdsworth, 1980). However, most experiments designed to investigate this question have tested the ability of a given agent to induce a release of calcium from isolated mitochondria which have previously been permitted to accumulate relatively large amounts (greater than 20 nmol/mg protein) of the cation (Borle, 1974; Peng, Price, Bhuvaneswaran & Wadkins, 1974; Binet & Volfin, 1975; Schotland & Mela, 1977; Lehninger et al., 1978; Harris et al., 1979; Lötscher et al., 1979; Roman et al., 1979). In comparison, the exchangeable calcium present in mitochondria in the cell is much lower (estimated to be about 1 and 2.5 nmol per mg of protein for mitochondria in kidney cells (Borle & Uchikawa, 1979) and liver (Claret-Berthon, Claret & Mazet, 1977), respectively. Moreover, the possibility that in many, but not all (Blackmore et al., 1979; Barritt et al., 1981) situations within the cell the adjustment of mitochondrial calcium concentration is a relatively slow process which is governed principally by the activity of the transporter which mediates calcium outflow (Akerman, 1980; Nicholls & Brand, 1980; Nicholls & Crompton, 1980) has not been fully considered in studies with isolated mitochondria.

These considerations point to the need for an experimental system in which the transport and accumulation of calcium by isolated mitochondria can be studied under conditions which approach more closely those of the cell cytoplasm. Three parameters appeared to be particularly important in this respect. These are a concentration of free extramitochondrial Ca^{2+} comparable to that present in the cell cytoplasm [about 0.01–0.1 μM (Baker, 1978)], the presence of extramitochondrial magnesium, and temperature. Previous experiments (reviewed by Bygrave, 1978; Carafoli, 1979; Fiskum & Lehninger, 1980; Nicholls & Crompton, 1980) have generally been conducted at high concentrations of free calcium, in the absence of magnesium and at temperatures below 37 °C. Magnesium is present in tissues at concentrations in the millimolar range, and Mg^{2+} has been shown to cause a significant increase in the value of $K_{0.5}$ for mitochondrial calcium uptake (reviewed by Bygrave, 1978). The quantity of calcium accumulated by mitochondria depends on the ratio of the rates of inflow and outflow which, in turn, are dependent (in part) on the rate of respiration. Since the rates of all three processes are temperature dependent (Heaton & Nicholls, 1976; Nicholls, 1978), temperature was considered to be an important factor which determines the amount of calcium accumulated.

Recently, the ability of isolated liver (Nicholls, 1978; Brand & De Selincourt, 1980) and brain (Nicholls & Scott, 1980) mitochondria to buffer the extramitochondrial calcium concentration at about 0.3–1 μM has been described. However, with one exception, all the experiments reported were conducted in the absence of magnesium. Moreover, these experiments provide no information on how the mitochondria respond to concentrations of calcium below the range 0.3–1 μM which may be imposed on them by the activity of the plasma membrane calcium transporters (Rasmussen & Gustin, 1978).

The aim of the present experiments was to develop and characterize a system in which the transport and accumulation of calcium by isolated liver mitochondria can be studied under conditions which approach those to which the organelles are exposed within the cell more closely than conditions employed in previous studies. The major features of the system chosen are a temperature of 37 °C, a concentration of extramitochondrial free calcium near 0.1 μM, and the presence of magnesium, Pi and ATP. A steady-state ^{45}Ca exchange technique was used to determine rates of calcium transport and the distribution of calcium between the medium and mitochondria. In contrast to the measurement of Ca^{2+} using an ion-selective electrode or spectrophotometric techniques, ^{45}Ca exchange can be employed under a wide variety of chemical environments. Together with the measurement of total extramitochondrial calcium by atomic absorption spectroscopy, this technique provides information about the quantities and kinetic properties of exchangeable calcium inside as well as outside the mitochondria.

The results have shown that under the conditions employed, two kinetically distinct compartments of exchangeable calcium are associated with isolated liver mitochondria. The kinetic properties of this exchangeable calcium are similar to those for cellular mitochondrial exchangeable calcium reported by others from studies conducted with perfused liver or isolated liver cells. In order to test whether the system is sufficiently sensitive to detect changes in the rates of calcium inflow or outflow, such as those which might be induced by potential mediators of hormone action, the effects of Ruthenium Red and nupercaine were investigated.

Materials and Methods

Isolation of Mitochondria

Mitochondria that sedimented at between 3,000 and 22,500 g/min ("heavy" mitochondria as defined by Prpić et al., 1978) were isolated as described by Hughes and Barritt (1978) with the modification

that the rats were anaesthetized with diethylether before removal of the liver. Mitochondrial respiration (using 12.5 mM succinate as substrate) and the protein content of mitochondrial suspensions (Biuret method) were measured as described previously (Hughes & Barritt, 1978). Values of 5–7 were routinely obtained for the ratio of the rates of ADP-stimulated/ADP-depleted oxygen consumption.

^{45}Ca Exchange

The reaction medium contained, in a final volume of 2–6 ml, 150 mM KCl; 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES[1])-KOH; 10 mM nitrilotriacetic acid; 2 mM potassium phosphate; 2 mM potassium succinate; 3 mM sodium ATP; 2 mM $MgCl_2$ (except where indicated otherwise) 10, 25, 50 or 100 μM added $CaCl_2$, as indicated; and 1.5 mg of mitochondrial protein per ml. The final pH was 7.4, or in some cases, 7.0 (as indicated). Except where indicated otherwise, incubations were conducted as follows in water-jacketed cylindrical incubation chambers situated on top of a magnetic stirrer. All reagents except the mitochondria and ^{45}Ca were mixed and allowed to reach 37 °C. The mitochondria were added, and after 15 min, tracer amounts of $^{45}CaCl_2$ (0.3–1.5 kBq per ml of incubation medium). At different times after the addition of the $^{45}CaCl_2$ (t(min), indicated on the Figures) samples (0.2 ml) of the incubation medium were withdrawn and mixed with 0.2 ml of quench medium which consisted of 0.5 mM EGTA, 150 mM KCl, 10 mM HEPES (pH 7.4) and 8 μM Ruthenium Red (Reed & Bygrave, 1975) in a plastic centrifuge tube at 0 °C. The tubes were centrifuged at $7,000 \times g$ for 2 min, the supernatants removed by aspiration, a further 0.1 ml of quench medium added, the tubes mixed briefly, centrifuged at $7,000 \times g$ for 1 min and the supernatant removed by aspiration. Each pellet was dissolved in 0.2% (wt/vol) sodium deoxycholate, and the amount of ^{45}Ca present determined using a liquid scintillation technique as described previously (Hughes & Barritt, 1978). The concentration of ^{45}Ca present in the incubation medium was also determined by liquid scintillation under similar conditions.

In order to determine the amounts of radioactivity associated with the extracellular fluid which surrounded the mitochondrial pellet, two samples (0.2 ml) were withdrawn from the incubation medium 2 min before the addition of ^{45}Ca. Each sample was mixed with 0.2 ml of quench medium which contained ^{45}Ca (in proportion to the final concentration of ^{45}Ca in the incubation medium), treated as described above, and the amount of ^{45}Ca associated with the mitochondrial pellet determined. The amount of radioactivity in these "zero time" reactions, which was generally less than 7% of the maximum amount of ^{45}Ca associated with the mitochondrial pellets, was then subtracted from the radioactivity present in each of the mitochondrial pellets obtained at time t (min).

It has previously been shown that the EGTA and Ruthenium Red present in the quench medium act to inhibit further ^{45}Ca exchange and remove $^{45}Ca^{2+}$ which is bound to EGTA-accessible sites on the outside of the mitochondria (Reed & Bygrave, 1975).

Analysis of ^{45}Ca Exchange Curves

The quantity of ^{45}Ca associated with the mitochondrial pellet (q_m) was expressed as a fraction of the initial dose of ^{45}Ca (q_{ao}). (A full list of symbols and their definitions is given in Table 1.) It was shown (*see* Results) that plots of q_m/q_{ao} against time t are described by the following equation:

$$q_m/q_{ao} = q_{41} - q_{42}\, e^{-\lambda_2 t} - q_{43}\, e^{-\lambda_3 t} \quad (1)$$

[1] *Abbreviations used:* HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Ca_T, total calcium, Ca_F^{2+} and Mg_F^{2+}, free (ionized) calcium and magnesium, respectively; and Pi, inorganic phosphate.

Table 1. Definition of symbols used to describe the kinetic parameters

Symbol	Definition
t	Time elapsed after the addition of ^{45}Ca to the mitochondrial suspension (min)
q_m	Quantity of ^{45}Ca associated with the mitochondria (Bq)
q_{ao}	Initial dose of ^{45}Ca added to the incubation medium (Bq)
q_m/q_{ao}	Quantity of ^{45}Ca associated with the mitochondria expressed as a fraction of the initial dose of ^{45}Ca
λ_n	Exponent (slope) in the nth term of the sum of exponentials equation describing the change in q_j with t
q_{jn}	Coefficient of the nth term in the sum of exponentials equation describing the change in q_j with t
a, b and c	Compartments of exchangeable calcium (represented schematically in Fig. 4)
Q_a, Q_b, Q_c	Quantities of exchangeable calcium in compartments a, b and c, respectively (nmol/mg of protein)
k_{ij}	Fractional transfer rate (rate constant) for the transport of ^{45}Ca or unlabeled calcium into compartment i from compartment j (min^{-1})
R_{ij}	Flux (amount of unlabeled and labeled calcium transported) into compartment i from compartment j per unit time (nmol/min per mg of protein)

where q_{41}, q_{42}, q_{43}, λ_2 and λ_3 are constants, and the initial conditions of the experiment dictate that $q_{41} - q_{42} - q_{43} = 0$. Eq. (1) and an equation which contains only one exponential term were fitted to the data by an iterative, nonlinear, least-squares procedure in which the standard deviations of the data were used for calculation of statistical weights (Berman, 1965). The SAAM 27 version of the computer program developed by Berman (1965) and a DECsystem 10 computer were employed for this purpose. The criteria used to assess goodness of fit were that (*i*) a minimum sum of squares was reached, (*ii*) the calculated values of q_m/q_{ao} did not deviate systematically from the observed data and (*iii*) variances for the estimated constants were low. The F ratio test described by Boxenbaum, Riegelman and Elashoff (1974) was used to assess whether the sums of squares obtained by fitting Eq. (1) to the data was significantly lower than that obtained for a fit of an equation which contained only one exponential term.

A three-compartment closed system (*see* Results) was fitted to the experimental data using the SAAM 27 computer program. The program calculates the values (±SD) of each of the four fractional transfer rates (initial dose of radioactivity in the medium, $q_{ao} = 1.0$). In turn, these values together with value of Q_a, the quantity of exchangeable calcium in the medium at equilibrium, are used to calculate the values (±SD) of the quantities of exchangeable calcium in the two mitochondrial compartments as well as the fluxes of calcium between the three compartments.

Estimation of Total and Free Calcium Concentrations

After removal of the mitochondria by centrifugation at $10,000 \times g$ for 5 min, the concentration of total calcium (Ca_T) in the extramitochondrial medium was measured by atomic absorption spectroscopy, using an air-acetylene flame, as described previously (Hughes

& Barritt, 1978). Standard calcium solutions were prepared in a medium of exactly the same composition. Calcium was extracted from mitochondrial pellets (4 mg of mitochondrial protein) with 2.0 ml of 0.3 M $HClO_4$ which contained 1 mM $LaCl_3$ (Blackmore, Brumley, Marks & Exton, 1978) at 0 °C. The amount of calcium present was measured (atomic absorption spectroscopy) using standard calcium solutions prepared in 0.3 M $HClO_4$/1 mM $LaCl_3$.

The concentrations of free Ca^{2+} and Mg^{2+} in the extramitochondrial medium were calculated from the measured concentrations of total calcium and magnesium with the aid of a computer program which is an algorithm of the program (Comics) developed by Perrin and Sayce (1967). The interactions between metal ions, protons and anions which were considered (pH of 7.4 and ionic strength of 0.39), together with the logarithm of the equilibrium constant (Sillen & Martell, 1971) in parentheses, were: HPO_4^{2-} with H^+ (6.73), Ca^{2+} (1.86), $H^+ + Ca^{2+}$ (7.60), Mg^{2+} (1.62); 2 nitrilotriacetic acid^{3-} with 2 H^+ (9.62), 2 Ca^{2+} (6.53), Ca^{2+} (9.96), 2 Mg^{2+} (5.37), Mg^{2+} (8.57); succinate^{2-} with H^+ (5.28), Ca^{2+} (1.2), Mg^{2+} (1.2); and ATP^{4-} with H^+ (6.52), Ca^{2+} (3.94), $H^+ + Ca^{2+}$ (8.65), Mg^{2+} (4.28), $H^+ + Mg^{2+}$ (8.81).

Materials

^{45}Ca was obtained from Amersham Australia Pty. Ltd., Sydney, New South Wales; nupercaine (dibucaine) hydrochloride from Ciba Pharmaceuticals, Crows Nest, N.S.W., Australia; and Ruthenium Red, which was recrystallized once before use, from the Sigma Chemical Co., St. Louis, Mo. All other reagents were of the highest grade available.

Results

Properties of ^{45}Ca Inflow Exchange Curves at 37 °C

The concentrations of added total magnesium (2 mM), phosphate (2 mM), ATP (3 mM) and H^+ (pH 7.4) initially employed in the incubation medium were chosen using the quantities of magnesium (Veloso, Guynn, Oskarsson & Veech, 1973), phosphate (Lawson, Guynn, Cornell & Veech, 1976) and ATP (Lawson et al., 1976; Siess, Brocks, Lattke & Wieland, 1977) together with the pH (Lawson et al., 1976) estimated to be present in liver tissue as a guide. Mitochondria were added to the incubation medium (in the presence of a given concentration of ^{40}Ca) 15 min before the addition of a tracer amount of ^{45}Ca. The curves which relate the amount of ^{45}Ca associated with the mitochondria, q_m (expressed as a fraction of the initial dose of ^{45}Ca, q_{ao}), to the time elapsed after addition of the ^{45}Ca at 50 and 25 μM added Ca_T are shown in Fig. 1*A* and *C*, respectively. All ^{45}Ca associated with the mitochondria was released by addition of either dinitrophenol (Fig. 2*A*) or antimycin A (Fig. 2*B*) in the presence of oligomycin.

Three types of experiment were performed in order to assess the stability of mitochondria during incubation at 37 °C. (*i*) The ratio of the rates of ADP-stimulated/ADP-depleted oxygen utilization (measured as described in Materials and Methods) was found to decrease from 4.7 ± 0.1 for mitochondria which had not been incubated at 37 °C to 3.7 ± 0.05 and 3.6 ± 0.3 ($n=2$) for mitochondria incubated at 37 °C for 15 and 25 min, respectively. (*ii*) no significant change in the initial rate of ^{45}Ca exchange was observed when the ^{45}Ca was added at 1, 2, 5, 10 or 15 min after initiation of incubation of the mitochondria with the medium (25 μM added Ca_T, results not shown). (*iii*) The shapes of ^{45}Ca exchange curves obtained when ^{45}Ca was added at 12, 18, 24 and 36 min after the mitochondria were compared (50 μM added Ca_T). No difference was observed for the curves obtained at 12, 18 and 24 min while the plateau of the curve obtained at 36 min was slightly lower (data not shown). These results indicate that the mitochondria are stable under the incubation conditions employed. This stability was dependent on the presence of ATP (results not shown).

The time taken for the system to reach equilibrium with respect to the net movement of calcium between the medium and mitochondria was investigated by measuring the concentration of extramitochondrial Ca_T (by atomic absorption spectroscopy). At 100 (Fig. 3*A*) and 25 μM (Fig. 3*B*) added Ca_T, the concentration of extramitochondrial Ca_F^{2+} decreased as the time of incubation increased, reaching a plateau of about 0.15 μM at 15 min. At 10 μM added Ca_T the concentration of extramitochondrial Ca_F^{2+} remained constant after an initial rise (data not shown). It was concluded that equilibrium is reached by 15 min.

The values obtained for the concentrations of total and free extramitochondrial calcium at 15 min for the four concentrations of added Ca_T tested at 0.05 mM Mg_F^{2+} are shown in Table 2. It can be seen that similar values were reached at 25, 50 and 100 μM added Ca_T. The observation that at 10 and 25 μM added Ca_T the measured concentration of extramitochondrial calcium is higher than that added is due to the presence of endogenous calcium in the suspension of mitochondria.

Further evidence that equilibrium is reached at 15 min was obtained from experiments in which ^{45}Ca was added to the incubation medium at the beginning of the incubation (at the same time as the mitochondria). Plots of the amount of ^{45}Ca associated with the mitochondria as a function of time at 50, 100 or 200 μM added Ca_T reached a plateau at 15 min after addition of the mitochondria (results not shown).

Analysis of ^{45}Ca Inflow Exchange Curves

When the expression $\log(q_{m(max)}/q_{ao} - q_b/q_{ao})$, where $q_{m(max)}$ represents the maximum amount of ^{45}Ca associated with the mitochondria, was plotted as a function of time for data obtained at each concentration of added Ca_T (0.05 mM Mg_F^{2+}) listed in Table 2, a biphasic plot was obtained in each case (plots for

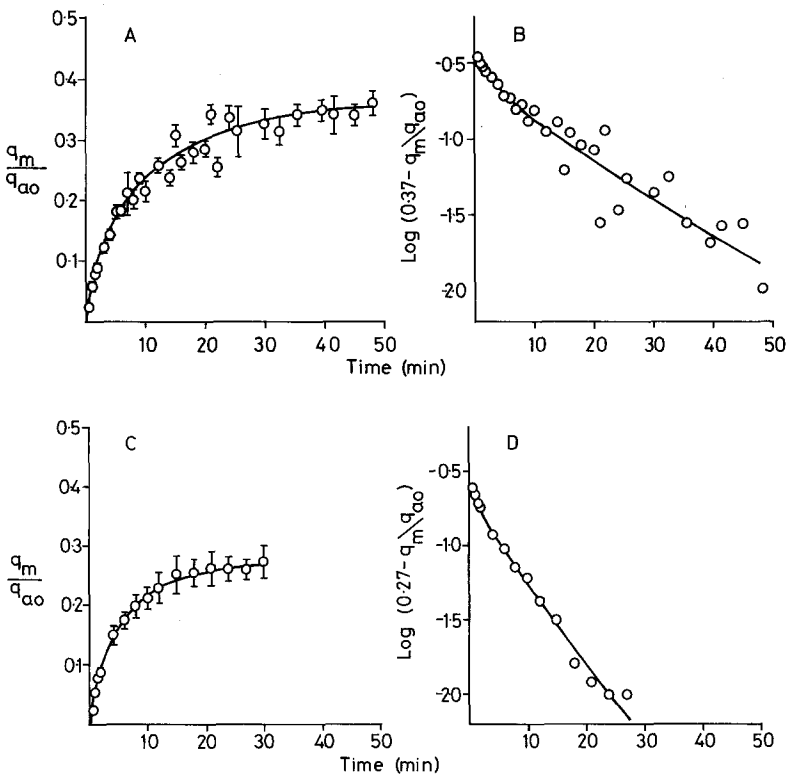

Fig. 1. ^{45}Ca inflow exchange curves for isolated liver mitochondria incubated in the presence of 3 mM ATP, 2 mM Mg_T (0.05 mM Mg_F^{2+}), 2 mM Pi and 50 (*A*, *B*) or 25 μM (*C*, *D*) added Ca_T. (*A*, *C*) Plots of q_m/q_{ao}, where q_m represents the quantity of ^{45}Ca in the mitochondria at time, t(min), and q_{ao} is the quantity of ^{45}Ca initially added to the medium, as a function of the time elapsed after addition of ^{45}Ca to the incubation medium. (*B*, *D*) Plots of $\log(q_{m(max)}/q_{ao} - q_m/q_{ao})$ as a function of time, where $q_{m(max)}/q_{ao}$ is the value of the plateau of the plot of q_m/q_{ao} against time. The incubation conditions, measurement of ^{45}Ca exchange and estimation of the quantity of ^{45}Ca associated with the mitochondria were as described in Materials and Methods. The results shown are the means ± SE of the combined results of 16 (*A*, *B*) or 5 (*C*, *D*) separate experiments. The solid lines were drawn using the values of the constants obtained from a fit of the three-compartment closed system (Fig. 4) to the data. Similar lines of best fit were obtained from a fit of Eq. (1) to the data

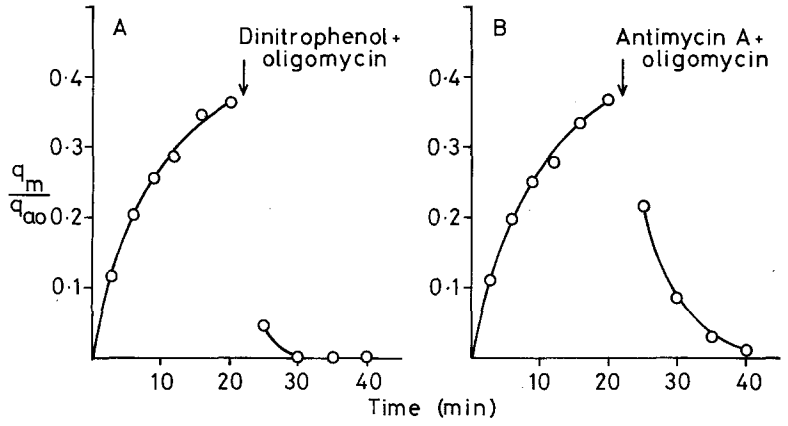

Fig. 2. The complete release of ^{45}Ca associated with the mitochondria by dinitrophenol and oligomycin (*A*) or antimycin A plus oligomycin (*B*). ^{45}Ca exchange was measured at 50 μM added Ca_T (0.17 μM Ca_F^{2+} at equilibrium) as described in the legend of Fig. 1. The concentrations of inhibitors employed were: dinitrophenol, 2 mM; antimycin A, 20 μM; and oligomycin, 45 μM. The results shown represent those from one of two experiments which gave similar results

data obtained at 50 and 25 μM added Ca_T are shown in Figs. 1*B* and 1*D*, respectively). Exponential equations were fitted to the data obtained at each of the four concentrations of added Ca_T in order to determine the form of the equation which gives the best fit to the data. An equation which contains two exponential terms [Eq. (1)] was found to be the simplest equation consistent with the data as judged by the criteria employed (*see* Materials and Methods). This result indicates that the ^{45}Ca exchange data can be described by a three-compartment closed system in which two compartments of exchangeable calcium are associated with the mitochondria (Shipley & Clark, 1972). Since the quantity of ^{45}Ca associated with the mitochondria does not include that bound to EGTA-accessible (extramitochondrial) sites, it is concluded that the two compartments of mitochondrial exchangeable calcium are located within the inner membrane. For further analysis of the data, it was assumed that these two compartments are arranged in a series configuration, as represented schematically in Fig. 4.[2]

[2] A parallel configuration of the three-compartment closed system also gave a good fit to the data obtained a 2 mM Mg_T (50 μM added Ca_T) and 10 mM Mg_T (10 μM added Ca_T). Although the values of the constants Q_b, Q_c, R_{ba} and R_{cb} obtained for these fits differ (maximum difference of about 100%) from those shown in Table 2, the major conclusions reached in this report do not depend on the assumption that the two compartments of mitochondrial exchangeable calcium are arranged in a series configuration.

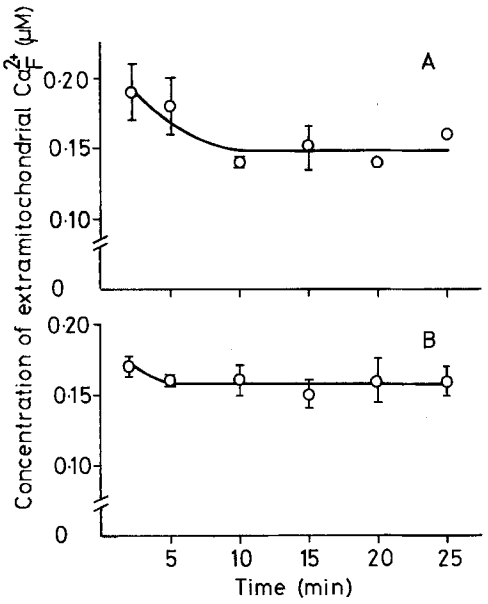

Fig. 3. The concentration of extramitochondrial Ca_F^{2+} as a function of the time elapsed after the addition of mitochondria to the incubation medium at 100 μM (*A*) and 25 μM (*B*) added Ca_T. The composition of the incubation medium, the conditions of incubation, and the method for the measurement of the concentration of extramitochondrial Ca_F^{2+} are described in Materials and Methods. The values shown are the mean ± SE (or range) of 1–4 separate experiments

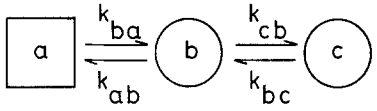

Fig. 4. Schematic representation of the three-compartment closed system found to be consistent with the data obtained from ^{45}Ca exchange experiments conducted with isolated rat liver mitochondria. Compartment (*a*) represents the extramitochondrial incubation medium and (*b*) and (*c*) are compartments of exchangeable calcium in the mitochondria. The constant k_{ij} (min^{-1}) represents the fractional transfer rate for the transport of calcium from compartment *j* to compartment *i*. Definition of the symbols used to describe the kinetic parameters are summarized in Table 1

The numerical values for the quantities of exchangeable calcium, fluxes and fractional transfer rates obtained for a fit of a three-compartment closed system (Fig. 4) to the four sets of data obtained at 0.05 mM Mg_F^{2+} are shown in Table 2. It can be seen that the values of the kinetic parameters associated with compartment (*b*) and the transfer of calcium between this compartment and the medium, compartment (*a*), are well-defined. However, the values of the constants for the transfer of calcium between compartments (*b*) and (*c*) (R_{cb}, k_{cb} and k_{bc}), which have high fractional standard deviations, are not so well-defined. Comparison of the sum of the quantities of exchangeable calcium in the mitochondrial compartments (Q_b+Q_c) with total mitochondrial calcium (measured by atomic absorption spectroscopy) indicates that a significant amount of calcium which does not readily exchange was only observed at 100 μM added Ca_T (Table 2).

Plots of the rate of calcium transport between the medium and compartment (*a*) as a function of the quantity of calcium present in each of these two compartments (Fig. 5) indicate that at values of extramitochondrial exchangeable calcium (Q_a) up to 25 nmol/mg protein (0.15 μM Ca_F^{2+}), calcium inflow to the mitochondria is not saturated (Fig. 5*A*). However, the data indicate that the process of calcium outflow from compartment (*b*) to the medium approaches saturation at the calcium concentrations employed (Fig. 5*B*). The quantity of exchangeable calcium in compartment (*b*) (Q_b) which gives half-maximal rate of transport was estimated to be 1.6 nmol/mg of protein and the maximum rate of calcium outflow 1.7 nmol/min per mg of protein (Fig. 5*B*).

Effects of Alterations in the Concentrations of Pi and Magnesium

When the concentration of Pi was increased from 2 mM to 4 mM, or decreased to 0.4 mM, no change in the exchange curve, or a 17% decrease in the plateau, respectively, were observed (results not shown). A decrease in the pH of the incubation medium from 7.4 to 7.0 and the replacement of succinate with pyruvate (5 mM) increased the plateau of the exchange curve by 80% and the time at which 50% of the ^{45}Ca had exchanged by 75% (data not shown).

The concentration of Mg^{2+} has been shown to be an important factor which determines the rate of calcium transport and the steady-state accumulation of calcium by mitochondria (reviewed by Bygrave, 1978; Becker, 1980; Becker, Fiskum & Lehninger, 1980). An increase in the concentration of total magnesium from 1 to 2 or 10 mM (0.02, 0.05 and 1.1 mM Mg_F^{2+}, respectively) decreased both the initial rate of ^{45}Ca exchange and the plateau of the exchange curve (Fig. 6). However, the change induced by a 20-fold increase in Mg_F^{2+} from 0.05 to 1.1 mM was much less than that induced by a three-fold increase in Mg_F^{2+} from 0.02 to 0.05 mM. This indicates that at 0.05 mM Mg_F^{2+}, the calcium transport systems are nearly saturated with magnesium.

The three-compartment closed system was also found to be the simplest compartment configuration which is consistent with the data obtained at 1.1 mM Mg_F^{2+}. The numerical values obtained for a fit of this configuration (Fig. 4) to the data are shown in Table 2. At 1.1 mM Mg_F^{2+}, the concentration of Ca_F^{2+} at equilibrium was found to be about three times

Table 2. Quantities of total and exchangeable calcium in the extramitochondrial and mitochondrial compartments, and the values and standard deviations of the kinetic constants for the transport of calcium between the three compartments of exchangeable calcium[a]

	Concentration of added Ca_T (μM)				
	10	25	50	100	10
Extramitochondrial magnesium concentration (mM)					
Total	2	2	2	2	10
Free	0.05	0.05	0.05	0.05	1.1
Quantity of extramitochondrial calcium at equilibrium					
Total (μM)	20 ±1.5	31 ±5	38 ±4	36 ±8	35 ±1.2
Free (μM)	0.08 ±0.006	0.13 ±0.02	0.16 ±0.02	0.15 ±0.03	0.42 ±0.01
Exchangeable (nmol/mg)					
Q_a	13 ±1	21 ±3	25 ±3	24 ±5	23 ±0.8
Quantity of mitochondrial calcium at equilibrium (nmol/mg)					
Total	5.7 ±1.0	9.5 ±0.1	16 ±1	43 ±6	ND[b]
Exchangeable					
Q_b	1.85 ±0.18	5.3 ±1.1	7.0 ±2.0	11.9 ±4.7	4.2 ±0.4
Q_c	1.3 ±1.7	2.8 ±0.9	7.5 ±1.9	12.7 ±3.5	4.2 ±1.7
Fluxes (nmol/min per mg)					
R_{ba}	0.59 ±0.02	1.30 ±0.05	1.47 ±0.16	1.37 ±0.16	1.2 ±0.04
R_{cb}	0.05 ±0.06	0.26 ±0.34	0.46 ±0.35	0.67 ±0.64	0.21 ±0.11
Fractional transfer rates (min^{-1})					
k_{ba}	0.045 ±0.002	0.062 ±0.003	0.059 ±0.007	0.057 ±0.007	0.051 ±0.002
k_{ab}	0.32 ±0.04	0.25 ±0.06	0.21 ±0.08	0.12 ±0.06	0.28 ±0.04
k_{cb}	0.03 ±0.03	0.05 ±0.07	0.07 ±0.07	0.06 ±0.08	0.05 ±0.03
k_{bc}	0.04 ±0.11	0.09 ±0.12	0.06 ±0.05	0.05 ±0.04	0.05 ±0.05

[a] Measurement of the concentrations of total calcium and calculation of the concentration of free extramitochondrial calcium and magnesium were conducted as described in Materials and Methods. ^{45}Ca inflow exchange experiments, analysis of the data and calculation of the values of the kinetic parameters (defined in Table 1) were performed as described in Materials and Methods and in the legend of Fig. 1. The values for total calcium and free and exchangeable extramitochondrial calcium are the means ± SD of 3 to 6 experiments performed at each concentration of added Ca_T. The values for the kinetic parameters were obtained from the analysis of the combined data from 4, 5, 16 and 7 experiments conducted at 2 mM total magnesium and 10, 25, 50 and 100 μM added Ca_T, respectively, and 4 experiments conducted at 10 mM total magnesium.

[b] ND, not determined.

that observed at 0.05 mM Mg_F^{2+}. However, the values for the kinetic constants obtained at 1.1 mM Mg_F^{2+} and 10 μM added Ca_T are similar to those obtained at 0.05 mM Mg_F^{2+} and 25 μM added Ca_T.

Effects of Ruthenium Red and Nupercaine

Ruthenium Red inhibits mitochondrial calcium inflow catalyzed by the electrophoretic uniporter but appears to have no effect on calcium outflow mediated by the proposed calcium outflow transporter (reviewed by Bygrave, 1978; Carafoli, 1979; Nicholls & Crompton, 1980). The effect of a low concentration of Ruthenium Red on ^{45}Ca exchange was to decrease the quantity of exchangeable mitochondrial calcium (Q_b+Q_c) and the flux R_{ba} (Table 3). The predominant effect on the fractional transfer rates was on k_{ba} (a decrease of 63%) with a smaller decrease (29%) in k_{ab} (Table 3). In other experiments conducted at the same or higher (4 or 16 nM) concentrations of Ruthenium Red at 25 or 100 μM added Ca_T, the predominant effect of the inhibitor on the fractional transfer rates was also found to be on k_{ba} rather than k_{ab} (results not shown).

The main effect of the local anaesthetic nupercaine, which has been shown to inhibit mitochondrial calcium outflow (Dawson, Selwyn & Fulton, 1979), was to increase the quantity of exchangeable calcium in compartment (*c*). This increase is associated with a decrease in the fractional transfer rate, k_{bc}, for the movement of calcium from compartment (*c*) to compartment (*b*). Similar results were obtained in other experiments in which the effects of 25 and 50 μM nupercaine on ^{45}Ca exchange at 25 μM added Ca_T were investigated (results not shown).

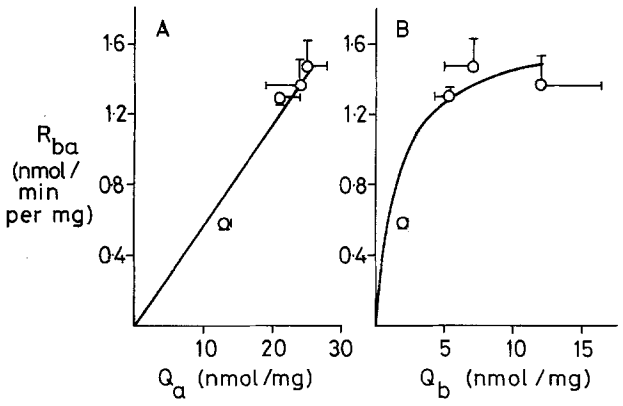

Fig. 5. The rate of transport of calcium (calcium flux) between mitochondrial compartment (*b*) and the extramitochondrial medium expressed as a function of the quantity of exchangeable calcium in the extramitochondrial medium, Q_a, (*A*) or compartment (*b*), Q_b, (*B*). The values for calcium flux R_{ba}, and the quantities of exchangeable calcium Q_a and Q_b (means ± SD) are taken from Table 2. The solid line in *B* has been drawn according to the equation $R_{ba}=1.7\ Q_b\ (1.6+Q_b)$ where 1.7 nmol/min per mg of protein and 1.6 nmol/mg of protein are the estimated maximum value of calcium flux and the value of Q_b which gives 50% saturation of calcium outflow, respectively. The solid line in *A* was drawn from a fit of the points by eye

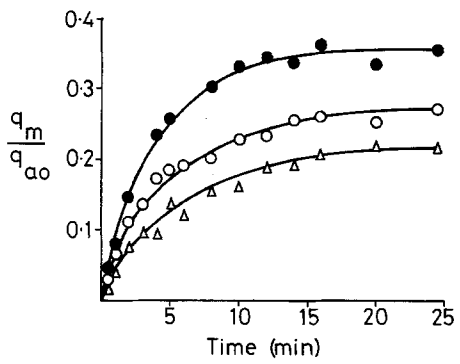

Fig. 6. Effect of alterations in the concentration of magnesium on ^{45}Ca exchange curves. The composition of the reaction media and measurement of ^{45}Ca exchange were performed as described in Materials and Methods. The concentrations of total magnesium were 1.0 (●), 2.0 (○) and 10.0 mM (△), and the estimated concentrations of free Mg^{2+} 0.02, 0.05, and 1.1 mM, respectively. The concentrations of added Ca_T were 25 μM (●, ○) and 10 μM (△). The data shown for each experimental condition are from one of four experiments which gave similar results. The solid lines were drawn from a fit of the data points by eye

Table 3. Effects of Ruthenium Red and nupercaine on kinetic parameters for calcium exchange determined from an analysis of ^{45}Ca inflow exchange curves obtained at 50 μM added total calcium and 2 mM total magnesium[a]

	Additions		
	None	Ruthenium Red (2 nM)	Nupercaine (50 μM)
Quantities of exchangeable calcium (nmol/mg)			
Q_b	9.2 ±2.0	5.5 ±2.4	8.5 ±1.0
Q_c	3.4 ±1.5	1.6 ±1.5	22 ±16
Fluxes (nmol/min per mg)			
R_{ba}	2.1 ±0.2	0.77 ±0.06	1.77 ±0.07
R_{cb}	0.4 ±0.6	0.5 ±1.0	0.5 ±0.2
Fractional transfer rates (min^{-1})			
k_{ba}	0.084 ±0.006	0.031 ±0.002	0.071 ±0.003
k_{ab}	0.23 ±0.06	0.14 ±0.07	0.21 ±0.03
k_{cb}	0.04 ±0.08	0.10 ±0.23	0.06 ±0.02
k_{bc}	0.11 ±0.19	0.33 ±0.35	0.02 ±0.02

[a] ^{45}Ca inflow exchange experiments, analysis of the results (the combined data for two experiments performed for each condition tested), and calculation of the values ± SD of the kinetic parameters (defined in Table 1) were performed as described in Materials and Methods. When present, the inhibitors were added at the beginning of the 15-min period of equilibration of the mitochondria with the incubation medium.

Discussion

In the present experiments two compartments, which contain about equal quantities of exchangeable calcium, were detected in isolated liver mitochondria. The fractional transfer rates for the outflow of calcium from the two compartments, about 0.1–0.3 and 0.04–0.09 min^{-1}, respectively, differ by a factor of two- to sixfold. The measurement of total mitochondrial calcium by atomic absorption spectroscopy revealed the presence of a third compartment of mitochondrial calcium which presumably exchanges very slowly in comparison to the time periods measured in the present ^{45}Ca exchange studies. At the highest concentration of added calcium employed, this very slowly exchangeable calcium accounted for about 50% of the total mitochondrial calcium.

The present report appears to represent the first time that this type of investigation of calcium distribution in isolated liver mitochondria has been undertaken. Most previous experiments have been directed towards measurement of the initial rate of calcium uptake under a variety of experimental conditions or the rate of calcium release under somewhat artificial conditions (the presence of EGTA or Ruthenium Red) (reviewed by Bygrave, 1978; Carafoli, 1979; Fiskum & Lehninger, 1980; Nicholls & Crompton, 1980). However, Studer and Borle (1980) have recent-

ly applied a similar ^{45}Ca exchange technique to studies of calcium distribution in isolated rat kidney mitochondria. These authors also found two compartments of mitochondrial exchangeable calcium, although in this case the fractional transfer rates for calcium outflow from the two compartments differ by a factor of about 30-fold.

The values of the fractional transfer rates for the outflow of calcium from mitochondrial compartments (*b*) and (*c*), k_{ab} and k_{bc}, respectively, observed in the present experiments conducted with isolated mitochondria are in reasonable agreement with those of 0.07 min^{-1} and 0.08 min^{-1} estimated for mitochondrial-exchangeable calcium from ^{45}Ca exchange studies conducted with perfused livers (Claret-Berthon et al., 1977) and isolated liver cells (Barritt et al., 1981), respectively. In these studies only one kinetically distinct compartment of exchangeable calcium which could be attributed to the mitochondria was detected (Claret-Berthon et al., 1977; Barritt et al., 1981). In the present study with isolated mitochondria it has proven reasonably difficult to clearly resolve two kinetically distinct compartments of mitochondrial exchangeable calcium. This may be partly due to the fact that the values of the fractional transfer rates differ by a factor of less than sixfold. Therefore it is possible that in the liver cell, which is a considerably more complex system than isolated mitochondria, the calcium exchange techniques employed to date have not been sufficiently sensitive to resolve two compartments of mitochondrial-exchangeable calcium.

The quantities of exchangeable calcium measured in the present experiments can be expressed in units of nmol per g wet wt of liver by making the assumption that 1 g wet wt of liver contains 50 mg of mitochondrial protein (Carafoli, 1967; Van Rossum, 1970). For the data obtained at 10 μM added Ca_T and 0.05 mM Mg_F^{2+}, the quantity of mitochondrial exchangeable calcium was estimated to be 0.16 nmol per mg wet wt of liver, and the rate of calcium transport, 0.03 nmol/min per mg wet wt of liver. These values are close to those of about 0.15 nmol per mg wet wt of liver and 0.02–0.03 nmol/min per mg wet wt of liver for these parameters, respectively, obtained from studies at 1.3 mM extracellular Ca_T with isolated liver cells (Barritt et al., 1981), and of 0.12 nmol per mg wet wt of liver for the quantity of mitochondrial-exchangeable calcium obtained from studies with the perfused liver (Claret-Berthon et al., 1977). These comparisons further support the argument that the incubation system described here is a reasonable one with which to study the transport and distribution of calcium in studies with isolated liver mitochondria.

In the presence of 10 μM added Ca_T and 1.1 mM Mg_F^{2+}, which is closer to the estimated physiological range of 0.6–1.3 μmol free Mg^{2+} per g wet wt of tissue (Veloso et al., 1973) than a concentration of 0.05 mM Mg_F^{2+}, values for the total amount of mitochondrial exchangeable calcium (Q_b+Q_c) and calcium flux across the mitochondrial inner membrane (R_{ba}) are about twofold greater than those obtained at 10 μM added Ca_T and 0.05 mM Mg_F^{2+}. However, at 1.1 mM Mg_F^{2+}, the equilibrium concentration of free Ca^{2+} in the extramitochondrial medium was higher by a factor of threefold. It is likely that if a lower concentration of extramitochondrial-free calcium were imposed on the mitochondria at 1.1 mM Mg_F^{2+}, lower values of (Q_b+Q_c) and R_{ba} would be observed.

The observations that (a) when presented with extramitochondrial Ca_F^{2+} at a concentration greater than 0.15 μM, mitochondria continue to take up calcium until the extramitochondrial Ca_F^{2+} concentration is reduced to about 0.15 μM, and (b) that under the conditions tested the calcium outflow pathway is close to saturation confirm, in a qualitative manner, two of the conclusions reached by Nicholls (1978) in experiments conducted with isolated liver mitochondria using a calcium-selective electrode. However, there are quantitative differences between the two sets of results. Thus, under the conditions employed by Nicholls (1978), the concentration of extramitochondrial Ca_F^{2+} maintained by the mitochondria was found to be higher (0.8 μM); and the maximum rate of calcium outflow was found to be 5 nmol/min per mg of protein compared with a value of about 1.7 nmol/min per mg of protein for R_{ba} observed in the present experiments. The routine use of magnesium in the present studies, and differences in pH, temperature and methods for the determination of the extramitochondrial free calcium concentration may account for these quantitative differences.

The conclusions reached from a comparison of the properties of exchangeable calcium in isolated mitochondria (present results) with those for mitochondria *in situ* (Claret-Berthon et al., 1977; Barritt et al., 1981) together with the results obtained with the inhibitors Ruthenium Red and nupercaine indicate that the incubation system and ^{45}Ca exchange technique employed in the present experiments can be used to detect changes in mitochondrial calcium inflow and outflow. It is concluded that the method is sufficiently sensitive to be used to investigate the effects of physiological changes (such as those which may result from the actions of hormones on cells) on the uptake and release of mitochondrial calcium.

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