The Uptake and Release of Calcium by Heart Mitochondria

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The kinetics of uptake of Ca^{2+} by rat heart mitochondria were studied by a spectrophotometric method with Arsenazo III indicator. The exponential rate coefficients measured with or without added phosphate increase with the amount of Ca^{2+} added up to about $24\ \mu\text{M}$. Evidence is given that the effect is attributable to a combination of formation of chelates at low concentrations to act as Ca^{2+} buffers, with co-transport of substrate to provide more respiratory fuel. The inhibitory effect of Mg^{2+} depends on the Ca^{2+} concentration, so with a constant $[\text{Mg}^{2+}]$ the low concentrations of Ca^{2+} are most inhibited, and the rate coefficients are still more Ca^{2+} -dependent. Ca^{2+} uptake is slowed by local anaesthetics such as butacaine and dibucaine, and also by propranolol and palmitoyl-CoA. After an uptake, the release of Ca^{2+} was investigated. The spontaneous release involves an initially slow and small appearance of free Ca^{2+} and is followed by an auto-accelerated phase. The release is accompanied by a gradual decrease in internal ATP; it is initiated by palmitoyl-CoA (reversed by carnitine), by lysophosphatidylcholine, by Na^+ salts (reversed by oligomycin) and by K^+ salts added to a K^+ -free medium containing valinomycin. The process is probably a response to an increased energy load imposed on the mitochondria by the various conditions, which include the spontaneous action of phospholipase activated by traces of Ca^{2+} . The problem of how much mitochondrial activity is participating in normal heart Ca^{2+} turnover is discussed, and experiments showing only 7–14% exchange of the mitochondrial Ca^{2+} occurring *in vivo* in 10 or 20 min are reported.

The rapid uptake of Ca^{2+} by mitochondria has attracted much attention, partly because there are so many related effects, which include stimulated respiration, shifts of internal and external pH, shifts of redox state (Chance, 1965; Lehninger, 1970), delocalization of respiratory components (Vinogradov *et al.*, 1972; Azzi *et al.*, 1975) and anion uptakes (Harris & Berent, 1969; Brand *et al.*, 1976). The practical question of physiological interest has been whether, and if so how, mitochondrial Ca^{2+} uptake supplements the activity of the sarcoplasmic reticulum in muscle, especially in the heart. Three studies have been described (Scarpa & Graziotti, 1973; Sordahl, 1975; Crompton *et al.*, 1976a), involving different conditions and methods, and not surprisingly giving differing values for cardiac mitochondrial Ca^{2+} fluxes. The present work adds more data to the picture and provides some comparisons of the effects of certain changes in conditions. An important but puzzling consequence of uptake of Ca^{2+} is that the time the cation is retained is limited and depends on many external factors. Some of these were described by Drahotka *et al.* (1965) in relation to the similar phenomena occurring with liver mitochondria. In the context of the heart, attention to the Ca^{2+} discharge has been intensified (Crompton *et al.*, 1976b; Carafoli *et al.*, 1974), because only by a cycle of uptake and non-traumatic release could the intracellular mitochondrion continue to regulate the free $[\text{Ca}^{2+}]$.

Evidence is presented that an oligomycin-sensitive energy load imposed by a low concentration of Na^+ added to the incubation mixture is the mechanism for the Ca^{2+} discharge described by Crompton *et al.* (1976b). The discharging action of palmitoyl-CoA appears to stem from its surfactant property of stimulating an ATPase,* since the effect is prevented by either provision of external ATP or adsorbing the compound on albumin. It is likely that factors such as low cytosolic ATP concentration, leakage of cytosolic protein, increase in Na^+ concentration and production of fatty acids and their derivatives as a consequence of Ca^{2+} -stimulated phospholipase activity all combine to cause failure of mitochondrial Ca^{2+} retention after a heart cell has been subject to anoxia or to a condition leading to excessive permeability (e.g. low Ca^{2+} concentration). It seems unlikely that mitochondrial activity plays much part in beat-to-beat Ca^{2+} cycling, if only because the rate of movement is highly sensitive to free Mg^{2+} .

Methods

Rat heart mitochondria were prepared by enzymic digestion of minced tissue as described by Harris & Zaba (1977). The final wash was given in a medium containing 250 mM-sucrose, 5 mM-Tris/HCl, 5 mM-KCl and 0.1% serum albumin at pH7. The mito-

* Abbreviation: ATPase, adenosine triphosphatase.

chondrial pellet was resuspended in the same buffered sucrose without the albumin and samples were taken for protein determination by the biuret method (Layne, 1957); then about 3 mg of albumin/ml was added. The Ca^{2+} uptake was followed in the double-beam spectrophotometer (Aminco-Chance DW2) by using purified Arsenazo III [2,7-bis-(*o*-arsonophenylazo)-1,8-dihydroxynaphthalene-3,6-disulphonic acid; Aldrich Chemical Co., Milwaukee, WI, U.S.A.] as indicator with the wavelength pair 685–675 nm with 30 nm slit. It was advantageous for high sensitivity to use a high concentration of the dye, but as the concentration is raised there is an appreciable effect of the formation of dye- Ca^{2+} complex on the rate of Ca^{2+} removal from the solution. Most experiments were made with sufficient dye to provide an A_{520} (the peak arsenazo absorption) of 0.5–1.5. Mitochondria were used at about 0.5 mg of protein/ml in a medium containing 120 mM-KCl, 20 mM-Tris/HCl, pH 7.2, 3.0 mM-Tris/succinate and 1 μg of rotenone/ml with additions noted in the text.

Experiments were also made in a vessel fitted with oxygen polarograph electrode and a Ca^{2+} -sensitive electrode (Radiometer, obtained from V. A. Howe Ltd., London S.W.6, U.K.). These allowed simultaneous records to be obtained of the respiration during prolonged retention of Ca^{2+} , and samples could be withdrawn at intervals for assay of ATP content and NAD^+ concentration in the medium after centrifugal separation (6000g-min) of the mitochondria with a Coleman microfuge. In this system, any required starting volume could be used; it was unsuitable for rapid kinetic work because the electrode response is slow, but the sensitivity is conveniently high when external $[\text{Ca}^{2+}]$ has been decreased to 1 μM or less by the mitochondria. The electrode reading was calibrated by using nitrilotriacetic acid/ Ca^{2+} mixtures (Reed & Bygrave, 1975).

Analyses for ATP in the acid extract of the mitochondria and for NAD^+ in a deproteinized supernatant were made by standard fluorimetric methods (Williamson & Corkey, 1969). Ca analyses were made by atomic absorption spectrophotometry (Techtron A.A. 100).

Results

Uptake of Ca^{2+}

The records from the spectrophotometer were corrected for the non-linear response of the indicator by use of a calibration curve; plots showed that the time course of the removal of 80% or more of the Ca^{2+} , then followed an exponential course. Hence a rate coefficient could be obtained by taking the inverse of the time taken for the external $[\text{Ca}^{2+}]$ to fall to $1/e$ (37%) of the total that was taken up. When no inhibitor of phosphate movement was present, the

total uptakes were close to the amounts initially added. Certain inhibitors, including those known to inhibit phosphate movement, decrease or abolish the uptake (Harris & Zaba, 1977). The rate coefficient observed was normalized to a standard protein concentration of 1 mg/ml by simple proportion. In fact, if the higher concentration of protein was used, uptake rate coefficients were less than proportionately increased; for example, at 0.4 mg/ml the observed value was 0.039 s^{-1} , corresponding to a normalized value of 0.097 s^{-1} , and at 0.8 mg/ml the observed value was 0.065 s^{-1} , corresponding to a normalized value of 0.081 s^{-1} . In practice, protein concentrations were kept within 0.4 and 0.6 mg/ml. The less-than-proportional dependence of rate on protein content parallels that observed for the phosphorylation of ADP (Harris, 1971), and may be due to aggregation of the particles. Measurements of rates by the method were not extended to concentrations about 50 μM because of the non-linear response; it appears preferable to use murexide, which is less sensitive but gives a linear response at higher concentrations of Ca^{2+} .

The rate coefficients of the uptake were measured both without added phosphate and with 3 mM added Tris phosphate. The results obtained at 22°C are shown in Fig. 1. The variability of the results indicated by the standard deviations on Fig. 1 and in the Tables arises mainly from differences between preparations, which in turn probably reflects the different proportions of active mitochondria making up the measured protein. The rate coefficient describing the removal of external Ca^{2+} increases with the initial concentration to which the Ca^{2+} is added over the range 1–24 μM . This can be restated as the influx being proportional to a power of the Ca^{2+} added, and so agrees with findings with liver mitochondria and with heart mitochondria in the presence of Mg^{2+} (Scarpa & Graziotti, 1973; Crompton *et al.*, 1976a). Rather than invoke 'co-operative action' between adjacent sites, which should surely operate during the course of removal of Ca^{2+} from the medium so as to provide successively lower rate constants, some alternative explanations were considered. One derives from the inescapable fact that mitochondrial suspensions leak soluble protein to the medium; such protein, like other proteins (serum albumin, for example), will bind more or less Ca^{2+} . According to the ratio of added Ca^{2+} to the adventitious protein, more or less of the total Ca^{2+} will be in the complexed form, so giving rise to a decreased free $[\text{Ca}^{2+}]$ which is, however, replenished as the complex dissociates. Hence, although a single exponential fits the uptake, the rate constant will be decreased as the ratio Ca/protein falls. Fig. 2 illustrates the consequence of adding serum albumin to the medium before recording the removal of an addition of 1 μM - Ca^{2+} . A test made with the albumin

Fig. 1. Concentration-dependence of rate coefficient of Ca²⁺ uptake

The rate coefficients of the exponential process that describes the removal of 80% of the Ca²⁺ added to the suspension of rat heart mitochondria. The initial Ca²⁺ concentration obtained by the addition is shown on the abscissa. Values are recalculated to a standard protein concentration of 1 mg/ml. (a) At 22°C, no added phosphate; (b) at 22°C, +3mm-phosphate; (c) at 32°C, +3mm-phosphate. The standard deviations from at least four experiments are shown on curves (a) and (b). Points on curve (c) are means of two observations.

alone showed that 1 mg in 2.5 ml halved the free Ca²⁺ concentration observed after an addition calculated to give 4 μM concentration.

A second factor likely to contribute to the dependence of the observed rate coefficient on the amount of Ca²⁺ added is the relative amount of co-transport of substrate anion. This effect contributes to the provision of energy for the uptake process. A co-transport of anions with Ca²⁺ has been described for liver mitochondria (Harris & Berent, 1969) and, by use of high (10–20mm) concentrations, it has been shown that hydroxybutyrate will accompany Ca²⁺ in

Fig. 2. Effect of albumin buffering on Ca²⁺ uptake. The records compare the courses of removal of Ca²⁺ added to provide 1 μM initial concentration (a) without and (b) with 0.3mg of serum albumin in 2.5ml of medium. The albumin lessens the free Ca²⁺ concentration and prolongs the uptake process. The medium was as described in the Methods section, with 3mm-Tris/phosphate at 22°C. The mitochondrial protein was 0.42mg/ml.

Table 1. Ca²⁺-induced uptakes of succinate by energized heart mitochondria

The succinate contents were corrected for the amount carried down non-specifically in the sucrose space (Harris & van Dam, 1968). The medium contained 120mm-KCl, 20mm-Tris/HCl, pH7.2, 3% dextran (w/v), 3mm-ascorbate, 50 μM-tetramethylphenylene-diamine hydrochloride and protein (2.5mg/ml) at 22°C.

External [succinate] (mm)	Ratio of succinate content (nmol/mg of protein)/external concn. (mm)	
	Before Ca ²⁺	After 12nmol of Ca ²⁺ /mg of protein
1.22	1.3	3.2
2.07	1.6	6.2

equivalent amount (Brand *et al.*, 1976). Table 1 shows that an appreciable amount of succinate is moved into heart mitochondria after an addition of Ca²⁺. That there is indeed some stimulation of the uptake process is most readily shown by comparing the rates found for successive additions of small quantities of Ca²⁺. Table 2 provides evidence that the rate coefficient for the second uptake exceeds that for the first. This result might reflect a general increase in permeability consequent on the first addition, but against this is the observation that the rate in a third test was often less than that in the second.

Effect of temperature

A set of measurements made in duplicate with one preparation at 32°C provided the data plotted as curve (c) in Fig. 1. There appears to be a higher temperature coefficient operative for additions of

Table 2. Rate coefficients (k) found for time courses of successive additions of Ca^{2+} recalculated to 1 mg of protein/ml

The medium was as described in the Methods section, at 22°C, with 0.67 mg of mitochondrial protein/ml.

First addition		Second addition		Third addition	
$[\text{Ca}^{2+}]$ (μM)	k (s^{-1})	$[\text{Ca}^{2+}]$ (μM)	k (s^{-1})	$[\text{Ca}^{2+}]$ (μM)	k (s^{-1})
4	0.082	20	0.110	4	0.096
4	0.090	20	0.099	4	0.099
4	0.081	20	0.099	4	0.093
20	0.085	20	0.116	20	0.110
24	0.082	24	0.096	24	0.090

concentrations above 20 μM than for those of lower concentrations, which perhaps reflects the limitation set by the buffering action of adventitious protein.

Composition of the medium

The inhibitory effect of Mg^{2+} on the movement of Ca^{2+} into heart mitochondria has been described (Sordahl, 1974; Jacobus *et al.*, 1975; Parr & Harris, 1976). The effect of MgCl_2 at 1 mM applied without added phosphate to a suspension at 22°C was to diminish the standardized rate coefficient (s^{-1} with 1 mg of protein/ml) for removal of Ca^{2+} added to a concentration of 24 μM from 0.085 ± 0.066 (S.D. for 12 results) to 0.047 ± 0.0063 ($n = 5$); with 3 mM-phosphate added, the respective rate constants were: 0.107 ± 0.013 ($n = 6$) and ($+\text{Mg}^{2+}$) 0.064 ± 0.0032 ($n = 6$). A range of Mg^{2+} concentrations was used in experiments done with additions of 24 μM -, 8 μM - and 1 μM - Ca^{2+} (Fig. 3), which showed that the inhibitory effect is limited, though considerable, and that it is reached at about 0.5 mM- Mg^{2+} . This Mg^{2+} concentration may approximate to that in the cytosol in some conditions (Randle *et al.*, 1968).

The rate coefficient for removal of Ca^{2+} applied at an initial concentration of 24 μM was measured in a set of media all containing 1 mM-Tris/phosphate and 20 mM-Tris/HCl and with KCl concentrations varied to alter the osmolarity. Over the range 60–190 mM-KCl the values were within 10% of each other, that is, no significant effect was found.

In another set of comparisons the replacement of the KCl by NaCl was tested. In the presence of 3 mM-Tris/phosphate and 1 mM- MgCl_2 there was no consistent difference but without the Mg^{2+} the uptake was slower in NaCl, in agreement with Carafoli & Crompton (1976).

The effect of pH change was tested over the range 6.75 ($k 0.065 \text{ s}^{-1}$), 7.0 ($k 0.084 \text{ s}^{-1}$), 7.2 ($k 0.077 \text{ s}^{-1}$), 7.5 ($k 0.073 \text{ s}^{-1}$) to 7.9 ($k 0.057 \text{ s}^{-1}$); these values were for removal of an initial addition of 24 μM - Ca^{2+} . The optimum pH appears to be between 7.0 and 7.2.

Fig. 3. Decrease by Mg^{2+} of Ca^{2+} -uptake rate coefficients

The rate coefficients describing uptakes of Ca^{2+} applied at (a) 24 μM , (b) 8 μM and (c) 1 μM to suspensions to which Mg^{2+} had been added. The medium was as described in the Methods section, with MgCl_2 addition. Temperature was 22°C. The mitochondrial protein was 0.5 mg/ml.

Rates measured in media with different succinate concentrations gave the following values as percentages of the value found with 6 mM-succinate: 0.6 mM, 30%; 1.2 mM, 65%; 1.8 mM, 86%; 3 mM, 110%. It was concluded that 3–6 mM-succinate was a suitable concentration range to use.

Inhibitors of Ca^{2+} uptake

The effects of two drugs, butacaine and dibucaine (nupercaine), were tested on the Ca^{2+} uptake process, because they had been found to be particularly effective for enhancing the response of Bromothymol Blue adsorbed to the mitochondrial protein towards Ca^{2+} (Chance *et al.*, 1968). With 3 mM-Tris/phosphate and an addition of 20 μM - Ca^{2+} the rate constant, normalized to 1 mg of protein/ml, was decreased by 80 μM -butacaine from 0.101 s^{-1} to 0.086 s^{-1} , and by 200 μM -butacaine to 0.059 s^{-1} . With 80 μM -dibucaine the value was 0.038 s^{-1} and with 120 μM -dibucaine it was 0.031 s^{-1} .

Propranolol is said to hinder the passage of Ca^{2+} through the cell membrane and it was found to hinder the Ca^{2+} translocation; the control rate (as above) was 0.101 s^{-1} ; this was decreased to 0.078 s^{-1} by 40 μM -propranolol and to 0.053 s^{-1} by 100 μM -propranolol.

Palmitoyl-CoA slows uptake of Ca^{2+} and promotes Ca^{2+} discharge by rabbit heart mitochondria

Fig. 4. Inhibitory action of palmitoyl-CoA on Ca²⁺ uptake

The curves show the course of removal of Ca²⁺, which was added to provide 10 μM in the medium. Curve (a) control; curve (b), +3 μM-palmitoyl-CoA; curve (c), +6 μM-palmitoyl-CoA. The medium was as described in the Methods section with 3 mM-Tris/phosphate at 22°C. The mitochondrial protein was 0.5 mg/ml.

(Asimakis & Sordahl, 1977). The inhibitory effect on uptake by rat heart mitochondria is illustrated in Fig. 4. In agreement with Asimakis & Sordahl (1977), L-carnitine lessens the inhibitory effect. This is presumably because the acyl group is transferred to carnitine and so is able to enter the cell. For example, the rate constant for uptake of an addition of 20 μM-Ca²⁺ in the presence of 3 mM-Tris/phosphate was 0.085 s⁻¹ without palmitoyl-CoA and this was decreased to 0.023 s⁻¹ by 12 μM-palmitoyl-CoA; with 2 mM-L-carnitine in addition the value was restored to 0.046 s⁻¹.

Release of Ca²⁺ and its initiation

After Ca²⁺ has been taken up there ensues a time during which the external concentration may be maintained at a value in the range 0.1–1 μM; the lower values are reached when serum albumin has been added. Without albumin after the Ca²⁺ uptake has ceased there follows a slowly increasing concentration of Ca²⁺ in the medium showing that retention of the accumulated ion is failing. The transfer back to the medium eventually auto-accelerates and all the original load of Ca²⁺ returns to the medium. The time for which the major part of the Ca²⁺ is retained depends on the ratio of added Ca²⁺/protein (Fig. 5), as for liver mitochondria (Drahota *et al.*, 1965).

Two approaches to the question of what controls the Ca²⁺ retention can be made; one is to seek correlated factors among the key metabolites, or the metabolic process itself, since energy supply is needed to retain the accumulated ions; the other is

Fig. 5. Dose-dependence of Ca²⁺-retention time

The removal of various additions of Ca²⁺ noted on the curves in nmol/mg of protein (at +Ca²⁺) from the medium and the later return of the Ca²⁺ to the medium as sensed by a Ca²⁺ electrode. Only the lower part of the record is shown; the peak [Ca²⁺] at the time of addition is less than the correct value because of the slow response. The electrode was calibrated with CaCl₂-nitrilotriacetic acid buffers. Note the log scale on the ordinate. The medium was as in the Methods section, with 1 mM-Tris/phosphate at 22°C. Protein was 1 mg/ml. The mixture was stirred and oxygenated.

to see what agents affect the retention in one or other direction.

In an experiment conducted like those of Fig. 5, samples were taken for assay of mitochondrial ATP content, and to find the concentration of nicotinamide nucleotide that had leaked into the medium. The results of the analyses along with a record of the readings of a Ca²⁺-sensitive electrode monitoring the Ca²⁺ concentration in the medium are in Fig. 6. There is a continuous loss of NAD⁺ and a decreasing internal ATP after the uptake of the Ca²⁺. Such consequences appear to be symptomatic of membrane damage rather than to be related to auto-accelerated Ca²⁺ release. A similar experiment (not shown), in which swelling and respiration were recorded, indicated that the swelling change occurred continuously, but it was at the time of rapid release of the Ca²⁺ that respiration slowed down. Again, these seem to be secondary effects.

As is well known for liver mitochondria (Drahota *et al.*, 1965), the presence of external ATP favours the retention of Ca²⁺ by the mitochondria. The same is true for heart mitochondria, and with ATP added to only 62 μM, the mitochondrial ATP after the Ca²⁺ uptake was 4.1 nmol/mg, and remained constant for

Fig. 6. Nucleotide changes during Ca^{2+} retention and loss. Samples were taken from a suspension containing 1.1 mg of protein/ml and centrifuged over an oil layer (Harris & van Dam, 1968) to obtain a supernatant for assay of NAD^+ and a mitochondrial extract for ATP measurement. Ca^{2+} was added at 110 nmol/mg of protein and further samples were taken at intervals. The Ca^{2+} electrode trace (a) shows the Ca^{2+} addition, though not the correct maximum concentration attained, and the change from uptake to loss at about 15 min; the loss has an auto-accelerated character. Values for NAD^+ lost to medium are at (b) and values for mitochondrial ATP content are at (c). The medium was as in the Methods section, with 1 mM-Tris/phosphate at 22°C; it was stirred and oxygenated.

the 22 min for which the experiment was continued (cf. Fig. 6). The other physiological agent that greatly affected the Ca^{2+} -retention time was serum albumin (cf. Azzone, 1966, for liver mitochondria). Added at 0.3 mg/ml to the suspension after equilibration with the medium, the free $[Ca^{2+}]$ fell by a factor of between 5 and 10; this seems to be a synergistic effect brought about by the albumin improving the mitochondrial coupling, because the respiratory rate is also diminished (as shown, for example, by the respective rates before Ca^{2+} addition noted in Fig. 7). If albumin is added after a quantity of Ca^{2+} , the retention time is prolonged, and even if shedding of

the Ca^{2+} has set in, it is reversed (Fig. 7). The protection by albumin against Ca^{2+} -induced damage points to phospholipase activity being involved. Mitochondria contain phospholipase (Waite & Sisson, 1971) and the enzyme is activated by low concentrations of Ca^{2+} (Nachbaur *et al.*, 1972). The albumin would both lessen the free Ca^{2+} concentration and bind fatty acids and lyso-compounds produced by lipase action. These compounds have themselves an uncoupling action; that of fatty acids is well known, and the effect of lysophosphatidylcholine, tested directly on Ca^{2+} retention, is illustrated in Fig. 8(a). The release of Ca^{2+} , which can be induced by a low concentration of palmitoyl-CoA (Asimakis & Sordahl, 1977), could be prevented by albumin. For example, where palmitoyl-CoA at 3 μM set off a Ca^{2+} release without albumin, in the presence of 0.13 mg of albumin/ml, 24 μM was required.

Another agent that would protect against palmitoyl-CoA, but not against lysophosphatidylcholine, was carnitine (Figs. 8b and 8a); presumably in the presence of carnitine the palmitoyl-CoA is rapidly removed by transferase activity. With carnitine added before the acyl-CoA, the Ca^{2+} release was not evoked (results not shown). ATP, whether added before the palmitoyl-CoA or after a limited amount of the acyl compound has evoked Ca^{2+} release (Fig. 8c), also has a protective influence. It appears that provision of a supply of energy to the membrane from the added ATP is countering an ATPase activity set up by the acyl compound.

The most interesting and potentially important way to initiate a discharge of Ca^{2+} is to add a Na^+ salt solution (Crompton *et al.*, 1976b; Carafoli *et al.*, 1974). This discharge can be of a limited quantity of Ca^{2+} , or can proceed to complete discharge, depending on the conditions. In Fig. 8(d) there is a limited discharge in response to 5 mM- Na^+ ; the addition causes an artifact because of dilution, and may also free some surface-bound Ca^{2+} . On adding oligomycin, there is a reversal of the Ca^{2+} movement. Fig. 8(e) shows traces obtained without and with ATP added to the medium; the response to the first addition of Na^+ without ATP is a limited Ca^{2+} release, which gives place to a sustained Ca^{2+} release when more Na^+ is added. With ATP present, the first addition of Na^+ causes some Ca^{2+} release, but further additions cause only a dilution artifact and very small and limited increments of free Ca^{2+} .

The protection afforded against release of Ca^{2+} given alternatively by oligomycin and by added ATP is explicable if the mechanism of Na^+ action is the setting up of an ATPase activity. Oligomycin would inhibit the ATPase, and external ATP would both provide a substrate and act to redistribute ATP between the particles with different rates of ATP production. The higher rate of respiration seen in

Fig. 7. Effect of albumin on the extent and duration of Ca²⁺ removal from the medium

The movements of Ca²⁺ are compared between suspensions (a) without added albumin (BSA) and (b) with 0.5 mg/ml. In (a) is also shown the consequence of adding albumin after the uptake of Ca²⁺ (80 nmol/mg of protein) had been nearly completed. The concentrations were sensed by a Ca²⁺ electrode (note the log scale); on account of the slow response, the maximum free Ca²⁺ produced by the addition is not recorded. Respiratory rates were measured simultaneously and are shown in parentheses (as ng-atoms of O/min per mg of protein). The medium contained 125 mM-KCl, 10 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.2, and 3 mM each of Tris/pyruvate, Tris/malate and Tris/phosphate at 22°C with 0.87 mg of protein/ml.

NaCl media compared with that in KCl media (Crompton *et al.*, 1976b) is consistent with the presence of a Na⁺-activated ATPase.

Graded discharge of Ca²⁺ evoked by K⁺ in the presence of valinomycin

The heart mitochondrion becomes permeable to K⁺ when exposed to valinomycin as shown by the exchange of ⁴²K and net movement of K⁺ (Harris *et al.*, 1966). Since K⁺ uptake consumes energy, it imposes a demand competing with that required to maintain a high ratio between internal and external Ca²⁺ concentrations. The situation can be equated to the induction of a K⁺-dependent ATPase.

When a low concentration of valinomycin is added to a suspension in an NaCl-based medium after an uptake of Ca²⁺ has occurred, a small amount of the Ca²⁺ is released (at V in Fig. 9). This can be ascribed to movement of K⁺ that had previously leaked into the medium. On addition of portions of KCl to the medium there are successive releases of Ca²⁺.

Exchanged Ca²⁺ in the mitochondria of animals given ⁴⁵CaCl₂ before removal of the heart

The possibility of inhibiting movement of Ca²⁺ with Ruthenium Red (Moore, 1971) provides a means of stopping transfer of labelled Ca²⁺ from cellular sources to the mitochondria during the

homogenization step of the preparation (Severson *et al.*, 1976). This method was applied to find the proportion of the Ca²⁺ in rat heart mitochondria that equilibrated with the plasma (containing a proportion of ⁴⁵Ca²⁺). Table 3 contains the values found after 10 or 20 min intervals between administration of the ⁴⁵CaCl₂ and removal of the heart; they are between 6 and 14% exchange. The results would be too low if recently taken-up Ca²⁺ had preferentially been lost during isolation of the mitochondria. With this reservation they point to a comparatively small participation of the mitochondrial Ca²⁺ in exchanges via the cytosol with the plasma Ca²⁺.

Discussion

The kinetic results are compared in Table 4 with data from other laboratories, one result among the latter being for rabbit heart mitochondria. The present values agree with those obtained by Crompton *et al.* (1976a), using the Mg²⁺-salt stopping procedure applied to ⁴⁵Ca²⁺ movement. An explanation has been proposed in the text for the observed [Ca²⁺]-dependence of the rate coefficient; it is noteworthy that Crompton *et al.* (1976b) found a hyperbolic dependence that, studied by one technique (Ca²⁺ electrode), became sigmoid in the presence of Mg²⁺. Crucial factors must be the amount of chelating material present (as shown in Fig. 2) and the quantity of Ca²⁺ in the media. Besides this potential to form

Fig. 8. Records illustrating the triggered release of Ca^{2+} and its reversal

The mitochondria had been loaded with 18 nmol of Ca^{2+} /mg of protein. Release in (a) was initiated by two additions of lysophosphatidylcholine (LPC) to provide a total of 8 μM and was not reversed by carnitine or oligomycin. In (b) release was obtained with 3 μM -palmitoyl-CoA (Pal-CoA) and was reversed by L-carnitine (6 mM). In (c) the consequence of the presence of ATP (40 μM) is compared with a trial in its absence; without the ATP the uptake still in progress was changed to a slow release by one addition of palmitoyl-CoA (6 μM), which became more rapid on a second addition (+6 μM), but was

Fig. 9. Stepwise release of Ca^{2+} in response to additions of KCl to a suspension in an NaCl-based medium after valinomycin has been added to induce K^{+} permeability. The mitochondria had been loaded with 33 nmol of Ca^{2+} /mg before making the additions. The medium contained 125 mM-NaCl, 20 mM-Tris/HCl, pH 7.2, 3 mM-succinate, 1 μg of rotenone/ml. The additions were: V, valinomycin (20 ng/mg of protein); K_1 , KCl to 5.5 mM; K_2 , KCl to 11 mM; K_3 , KCl to 16.5 mM.

Table 3. Ca^{2+} exchanged in rat heart mitochondria in vivo

Rats were injected intraperitoneally with about 50 μCi of $^{45}\text{CaCl}_2$. Mitochondria were prepared from the hearts with 5 μM -Ruthenium Red in the solution used for homogenization. The Ca^{2+} content and the $^{45}\text{Ca}^{2+}$ radioactivity in acid extracts of the mitochondria were measured. A sample of plasma was deproteinized with HClO_4 (0.5 M final concn.), and the Ca^{2+} concentration and $^{45}\text{Ca}^{2+}$ activity were determined so as to relate the radioactivity to total Ca^{2+} . By using this specific radioactivity the proportion of the mitochondrial Ca^{2+} , which had the same specific radioactivity as plasma Ca^{2+} , was calculated.

Time between $^{45}\text{Ca}^{2+}$ injection and removal of heart (min)	Total Ca^{2+} (nmol/mg of protein)	Ca^{2+} exchanged with $^{45}\text{Ca}^{2+}$ (%)
10	7.8	8.8
	13.0	13.9
	7.1	7.0
20	6.5	6.0
	10.2	12.6

stopped or reversed by ATP. With ATP present in the suspension, the first Pal-CoA (6 μM) addition produces only an artifact, the second (+6 μM) stops further Ca^{2+} uptake and the third (+6 μM) starts appreciable release. In (d) the Na^{+} -initiated release of a limited amount of Ca^{2+} is shown along with (from a second experiment) the re-accumulation induced by oligomycin (6 μg /mg of protein). In (e) the responses to additions of Na^{+} salt (all 5 mM) are compared with and without ATP (40 μM). Without ATP the first Na^{+} addition releases a limited amount of Ca^{2+} and the second Na^{+} addition induces a continuous release, whereas with ATP present the first Na^{+} addition displaces some Ca^{2+} , but only slight changes are seen at the second, third and fourth additions. In a trial without mitochondria it was found that 40 μM -ATP decreased the response to 0.4 μM - Ca^{2+} by only about 10%, so the effects in (c) and (e) are not solely due to chelation.

Table 4. Comparison between results obtained for Ca²⁺ influx by various methods
The fluxes are calculated or interpolated for an addition of 24 μM.

Conditions			Species	Flux (nmol of Ca ²⁺ /s per mg)	Method	Reference
Added		Temperature (°C)				
P _i (mM)	Mg ²⁺ (mM)					
2	5	26	Rat	1	Spectroscopic with murexide	Scarpa & Graziotti (1973)
8	0	30	Rabbit	1.2	Spectroscopic with murexide	Sordahl (1975)
0	0	25	Rat	0.35	Mg ²⁺ -stopping of ⁴⁵ Ca ²⁺ uptake	Crompton <i>et al.</i> (1976a)
0	0	25	Rat	0.32	Ca ²⁺ electrode	
1	0	25	Rat	2.7	Mg ²⁺ -stopping of ⁴⁵ Ca ²⁺ uptake	
1	1	25	Rat	0.43	Ca ²⁺ electrode	
0	0	22	Rat	2.0	Spectroscopic with Arsenazo III	This paper
3	0	22	Rat	2.7		
3	0	32	Rat	4.3		
0	1	22	Rat	1.1		
3	1	22	Rat	1.5		

more or less Ca-protein complex to act as a buffer, there is the observation that a second uptake can proceed at a higher rate than the first (Table 2), which points to a priming action explicable by co-transport of substrate. This co-transport exactly resembles that described for movement of substrate with K⁺ when valinomycin is present to induce a high K⁺ permeability (Höfer *et al.*, 1967). It seems likely that without appreciable buffering and with adequate substrate supply to satisfy the demand imposed by Ca²⁺ movement, the rate coefficient is initially constant and then decreases as the load imposed by the Ca²⁺ movement cannot be met by substrate supply. Put otherwise, the hyperbolic relation between Ca²⁺ flux and Ca²⁺ concentration is likely to be correct in the absence of Mg²⁺ (in agreement with Crompton *et al.*, 1976a). This does not, however, mean that in the cell the free Ca²⁺ will rapidly diminish in concentration owing to mitochondrial activity, because dissociation of the cytosolic Ca²⁺-buffering complexes with protein and ATP will be important. It has been estimated that 25–50 nmol of Ca²⁺ move per g of heart tissue at each beat (Crompton *et al.*, 1976a), and in the rat this is repeated 150 times per min. If we ask how much Ca²⁺ will move into the 80 or so mg of mitochondria per g of tissue (Scarpa & Graziotti, 1973) in 0.4 s at 37°C in the presence of 1 mM-Mg²⁺ and excess phosphate, it is first necessary to assume the concentration of free Ca²⁺ reaching the particles; with 1 μM-Ca²⁺ the mitochondrial uptake is of the order of 1 nmol per g of tissue, with 8 μM it is 10 and with 24 μM it is about 60. The reason why the amount increases more than in proportion to the [Ca²⁺] is because the rate constant in the presence of Mg²⁺ is Ca²⁺-dependent (Fig. 3; see also Scarpa & Graziotti, 1973; Crompton *et al.*, 1976a). To assess the im-

portance of the mitochondrial activity evidently the free [Ca²⁺] must be known. The Ca²⁺-exchange experiments reported in Table 3 show that only 6–12% of the Ca²⁺ found subsequently in isolated mitochondria has become exchanged with plasma Ca²⁺. If indeed there is little turnover *in vivo* in 10–20 min, the conclusion would be that only low free Ca²⁺ concentrations arise in the cell.

As pointed out by Chance (1965), it is important to ask how such Ca²⁺ as is taken up is released again. The agents that evoke Ca²⁺ release share the property of imposing an energy load on the mitochondria; the fatty acids and their derivatives by uncoupling, Na⁺ by stimulating an ATPase, and valinomycin by diverting energy to K⁺ uptake. Sordahl (1975) has shown that added ADP can lead to release of part of the accumulated Ca²⁺ from rabbit heart mitochondria. Hence it appears that any parallel load imposed on energy production can lead to a release of some of the Ca²⁺, which in turn implies that the rate of other energy-consuming processes in the cell will lead to adjustments in the concentration of free cytoplasmic Ca²⁺.

It is likely that one of the reasons why a low concentration of external ATP so effectively protects against Ca²⁺ release is that it provides a way of redistributing an energy source for Ca²⁺ uptake between particles whose ATP production is impaired, and those whose phosphorylation capacity is high. It would be important to maintain the Ca²⁺-accumulating ability of the whole population in order to prevent the auto-accelerated process of Ca²⁺-stimulated phospholipase acting on the de-energized particles to provide an increasing quantity of fatty acids and lyso-compounds. These compounds would attack the membranes of the neighbouring unimpaired particles. An argument that indeed there

are different grades of particles possessing a scale of sensitivities to the agents that release Ca^{2+} stems from the observation that release of more Ca^{2+} can be elicited by a further addition of Na^+ , for example, to a mixture already containing some Na^+ salt, and which has already liberated some Ca^{2+} to the medium. It is likely that less active mitochondria succumb to the extra load imposed by the first Na^+ addition, unless they are replenished by ATP shuttling from the more active ones (which requires external ATP). The protection against Na^+ release afforded by oligomycin is consistent with the inhibition of ATPase activity by the latter. How the antagonism between loading factors such as Na^+ and fatty acids in the cytosol are balanced against ATP and protein concentrations is a question that only further studies can answer. In general, it is obvious that trauma giving rise to low ATP and leakage of cellular protein will expose the mitochondria to damage from Ca^{2+} .

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