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THE ISOLATION OF COUPLED MITOCHONDRIA FROM *PHYSARUM POLYCEPHALUM* AND THEIR RESPONSE TO Ca^{2+}

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Summary

A method for the isolation of coupled mitochondria from the acellular slime mould *Physarum polycephalum* is described. The mitochondria oxidize respiratory substrates at rates comparable to those of mitochondria from other microorganisms and show similar responses to respiratory inhibitors. ADP/O values approach similar values to those obtained with mitochondria from higher organisms: 3 with NAD-linked substrates, 2 with succinate, and 1 with ascorbate-TMPD.

Mitochondria actively take up low concentrations of Ca^{2+} with stimulation of their respiration. With succinate or pyruvate-malate as substrates respiratory responses are depressed by Ca^{2+} concentrations in excess of 200 μM in the presence or absence of phosphate.

Exogenous NADH is unique in supporting the uptake of large amounts of Ca^{2+} in the presence of phosphate and in showing an unusual 'uncoupled' response in the absence of phosphate.

A sigmoidal relationship occurs between initial velocity of Ca^{2+} uptake and Ca^{2+} concentration with a maximum velocity of approx. 15 nmol/s per mg protein and half maximum velocity occurring at approx. 50 μM Ca^{2+}.

Introduction

Aspects of eukaryotic cell motility, in particular protoplasmic streaming, migration, and mitosis, are strongly expressed or amplified phenomena in

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*,2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N'*-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N*,*N*,*N'*,*N'*,-tetramethyl *p*-phenylenediamine hydrochloride.

plasmodia of the acellular slime mould, *Physarum polycephalum*. Actin and myosin have both been isolated from *Physarum* [1,2], and these proteins have properties generally similar to those found in most other eukaryotic organisms. Certain regulatory proteins thought to be associated with the actomyosin complex have also been described in *Physarum* [3]. For these reasons, *Physarum* has become an important model organism in which to study the molecular basis of non-muscle contractility, and the organisation and regulation of the contractile systems associated with the various motile functions of non-muscle cells.

It is thought that Ca^{2+} regulates interactions between actin and myosin in *Physarum*, either through actin-associated regulatory proteins, or by way of a direct effect on myosin [4,5]. Motile functions such as protoplasmic streaming [6,7] and mitosis [8] are associated with altered concentrations and fluxes of Ca^{2+} in *Physarum* plasmodia. In higher organisms, cytoplasmic Ca^{2+} levels are regulated by Ca^{2+} pumps in mitochondria [9], the endoplasmic reticulum [10], and the plasma membrane [11]. In *Physarum*, experiments with a microsomal fraction have indicated that this may contain a high affinity Ca^{2+} uptake system [12]. As yet there have been no reports that mitochondria or the plasma membrane contain such Ca^{2+} transport systems. In another lower eukaryote (*Saccharomyces*) physiologically significant mitochondrial Ca^{2+} transport systems are thought to be absent [13].

A survey of mitochondria isolated from plant tissues revealed that a majority of the mitochondria examined also lacked high affinity Ca^{2+} transport systems [14].

Previous attempts to isolate coupled mitochondria from *Physarum* have not been successful [15,16]. In view of the importance of *Physarum polycephalum* as a model system for cell cycle and cell motility studies, and the possible regulatory role of Ca^{2+} in these processes, we have devised a method for isolating active, coupled mitochondria from *Physarum* and have examined their response to Ca^{2+}.

Materials and Methods

Organism and culture conditions. Physarum polycephalum, strain M_3cV_{AD} was kindly supplied by Dr. K. Babcock of the MacArdle Laboratory for Cancer Research, University of Wisconsin, Madison, U.S.A. Microplasmodia were routinely grown in the medium of Carlile [17] with the modifications that $CaCl_2$, EDTA, biotin, and thiamine were omitted, and 0.15% yeast extract was included. This medium contains 0.25—0.3 mM Ca^{2+}, as assayed by atomic absorption spectrophotometry.

Measurement of respiration. Oxygen uptake was measured polarographically at 25°C with a Clarke-type electrode and Gilson Oxygraph. The saturation concentration of oxygen in 0.4 M mannitol, 10 mM KH_2PO_4, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.2, the buffer used for respiration measurements, was assumed to be 213 nmol/ml [18]. The oxygen concentration in other buffers was estimated by reference to this value. Total volume used in respiration measurements was 2.1 ml.

Estimation of protein. Mitochondria were diluted in buffered mannitol and centrifuged for 2 min in an Eppendorf centrifuge. The pellets were dissolved in

0.4 M NaOH, and aliquots assayed by the method of Lowry et al. [19].

Determination of ADP/O values and acceptor control ratios. ADP/O values were determined polarographically [20] and acceptor control ratios according to Chance and Williams [21].

Assays of subcellular fractions. Succinate dehydrogenase activity was assayed by the method of Singer [22].

Cytochrome spectra. Cytochrome difference spectra (hydrogen peroxide oxidized versus sodium dithionite reduced) were measured at liquid nitrogen temperature (−196°C) using a Cary 14R spectrophotometer with scattered transmission accessory.

Density gradient centrifugation. 30—65% (w/v) sucrose gradients in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, were prepared and centrifuged at 2°C in a Beckman SW27 rotor at 60 000 × *g* for 2 h. 1-ml fractions were collected through the bottom of the tube and assayed for protein concentration and enzyme activity.

$^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake by mitochondria was measured in a constantly stirred, water-jacketed vessel at 25°C. The basic uptake medium contained 0.4 M mannitol, 10 mM MES (pH 6.2) and 0.1 mM $^{45}CaCl_2$ (0.1 μCi/ml, 2.5 mCi/μmol) and the volume of the total incubation mixture was 2 ml. 100-μl samples were withdrawn, rapidly filtered through a 0.45 μm membrane filter and washed with 10 ml 0.4 M mannitol, 10 mM MES (pH 6.2), 100 mM KCl, 20 mM $MgCl_2$. Filters were dried and their radioactivity estimated by liquid scintillation counting.

Materials. Ruthenium red (Sigma) was purified before use by the method of Fletcher et al. [23]. $^{45}CaCl_2$ was obtained from the Radiochemical Centre, Amersham, U.K.

Results and Discussion

1. Factors affecting the preparation of actively respiring and phosphorylating mitochondria.

Four aspects of the isolation of mitochondria from *Physarum* were identified as critical in determining the ultimate quality and activity of preparations:

(a) Harvesting and washing of microplasmodia. When microplasmodia were chilled or left as pellets for any length of time, or washed with water after harvesting, their respiratory activity declined to near zero. It was noted that of all the washing procedures used those which caused the release of the smallest amounts of yellow pigment from the plasmodia also preserved respiration most effectively. As a consequence, after quickly sedimenting at room temperature, the microplasmodia were either washed at room temperature in 20 mM sodium citrate buffer (pH 4.6) or not washed at all.

(b) The disruption of filaments attached to mitochondria. We have observed an extensive fibrillar network in plasmodia which may interconnect subcellular organelles (Homes, R.P., Irving, D.O. and Stewart, P.R., unpublished). To disrupt this network chemically and thus prevent damage that might result from physically shearing these attachments, microplasmodia were incubated in a high salt buffer (0.6 M KCl) for 30 min before homogenization. Such an

incubation significantly increased the acceptor control ratios of isolated mitochondria.

(c) Homogenizing conditions. The use of relatively high osmolarity buffer; the substitution of metabolically inert mannitol for sucrose; the combination of a non-electrolyte with KCl; the presence of the Ca^{2+}-chelating agent EGTA; buffering at near neutral pH; the avoidance of strong-shearing conditions during homogenization; the use of low centrifugal forces to pellet the relatively large mitochondria from this organism; all of these factors were found to be important in the isolation of mitochondria with little endogenous respiration, but which actively oxidized tricarboxylic acid cycle intermediates with good acceptor control.

The need to include a high concentration of EGTA (5 mM) in the homogenizing medium indicates that *Physarum* mitochondria are sensitive to Ca^{2+}, a point which is taken up in further detail below. We have found that the homogenization of microplasmodia results in the release of 40% of the cellular Ca^{2+} in a diffusible form (Holmes, R.P. and Stewart, P.R., unpublished). The exposure of mitochondria to localized, high concentrations of Ca^{2+} that must occur during homogenization, could irreversibly damage them unless a Ca^{2+}-chelating agent is present. It should be noted that EGTA or EDTA are included in the majority of media used in the isolation of mitochondria from microorganisms, suggesting that these mitochondria are also sensitive to Ca^{2+} [26]. The pH of the isolation medium finally adopted (6.8) is close to the intracellular pH of *Physarum*, which fluctuates between 6.0 and 6.6 during the cell cycle [31]. Decreasing the pH of the isolation medium below 6.8 produced aggregation of cytoplasmic particles altering the composition of the mitochondrial fraction and its respiratory response.

(d) Stability of mitochondria following isolation. Physarum mitochondria prepared in simple salt or sugar buffers declined quickly in activity. Losses of up to two-thirds of the initial respiratory activity in the first 60 min after isolation were observed when preparations were held on ice. The combined use of bovine serum albumin and a thiol reagent such as cysteine or dithiothreitol was found to prevent this ageing affect; no significant losses of respiration were seen in 3 h at 0°C when mitochondria were washed and resuspended in the presence of these agents. Good quality commercial albumin (Fraction V, Sigma) was as effective in this respect as the same material defatted with activated charcoal [24]. Whether its protective effect is a consequence of the complexing of fatty acids [25] was not explored further.

2. Method adopted for isolation of mitochondria

After examining in some detail each of the factors mentioned above, the following method was evolved for routine preparations as representing the optimum combination of these various parameters.

Microplasmodia were harvested from log phase cultures by centrifuging at room temperature for 30 s at 250 × *g*. The pellet was resuspended in 2 vols. ice-cold, high salt buffer, containing 0.6 M KCl, 5 mM EGTA, 1% bovine serum albumin, 1 mM dithiothreitol, 10 mM MES, pH 6.8, and constantly stirred for 30 min at 0°C. The suspension was then diluted with 5 vols. ice-cold homogenizing medium which contained 0.4 M mannitol, 1% albumin, 5 mM EGTA,

1 mM dithiothreitol, 10 mM MES, pH 6.8, and disrupted at 4°C in a Thomas Type B tissue grinder by two strokes with a motor driven Teflon pestle rotating at 700 rev./min. All subsequent steps were carried out at 0—4°C using ice-cold solutions.

The homogenate was centrifuged at 1000 X *g* for 2 min, and the pellet containing mainly microplasmodial fragments discarded. The mitochondrial fraction was obtained from the supernatant by centrifuging at 3000 X *g* for 6 min. The brownish pellet (with only a trace of yellow pigment) was washed once in homogenizing medium which contained no EGTA but was supplemented with 0.1 M KCl. The yield of protein in this fraction was 5—7% of the protein in the homogenate.

Examination by phase-contrast microscopy showed that these preparations contained mitochondria (1—1.5 μm) predominantly, with a few membranous fragments and some swollen nuclei present. Further purification of mitochondria on a linear sucrose gradient indicated that mitochondria accounted for 70% of the protein in the fraction. The succinate dehydrogenase activity of purified mitochondria was 2.3 μmol/min per mg protein compared with an activity of 0.5 μmol/min per mg in the homogenate. This suggests that mitochondria could account for up to 20% of the plasmodial protein. The majority of the contamination in the mitochondrial fraction could be removed by pelleting the mitochondria through a 12.5% Ficoll cushion. However, such mitochondria were functionally inferior, possibly because of the high centrifugal force (15 000 X *g*) needed to pellet the mitochondria through the viscous Ficoll.

3. Oxidative phosphorylation by mitochondria

Mitochondria prepared by the method described above show low respiratory activity in the absence of substrate (Fig. 1). With substrate present, rates of

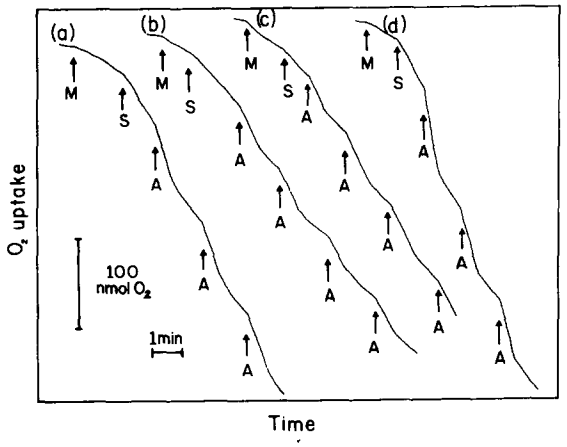

Fig. 1. Respiration of mitochondria with different substrates and the stimulation of respiration by ADP. M denotes the addition of mitochondria (0.7—1.2 mg), S the addition of substrate which in (a) gives a final concentration of 5 mM succinate, (b) 5 mM pyruvate + 0.5 mM malate, (c) 5 mM α-ketoglutarate, and (d) 1 mM NADH, and A the addition of 150 nmol of ADP, except in (d) where 300 nmol additions of ADP were made.

TABLE I

MEASUREMENTS OF RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN *PHYSARUM* MITOCHONDRIA

The results (except for α-ketoglutarate) are expressed as the mean values ± S.E. of the mean for 3 different preparations. The standard errors for acceptor control ratios and ADP/0 values were 0.1 or less.

Substrate	State 3 respiration rate (nmol/min per mg protein)	Acceptor	ADP/0
Succinate 5 mM	185 ± 25	1.7	1.7
NADH 1 mM	168 ± 19	2.6	2.6
Pyruvate 5 mM + Malate 0.5 mM	114 ± 18	2.6	2.2
α-Ketoglutarate 5 mM	112	1.9	2.0
Ascorbate 5 mM + TMPD 10 μM	89 ± 8	1.4	0.9

oxygen consumption are obtained which are comparable to those described for other lower eukaryotes (Table I; cf. Lloyd [26]). Multiple additions of low concentrations of ADP (75 μM) produce corresponding stimulations of respiration followed by a return to the prestimulated rate (Fig. 1). Acceptor control ratios and ADP/O values calculated for different substrates are summarised in Table I. In general, the values are comparable to those obtained with mitochondria from other microorganisms [26]. The ADP/O ratios approach values described for higher organisms.

In common with mitochondria from other microorganisms [26], exogenous NADH is oxidized. If *Physarum* mitochondria are similar to *Saccharomyces* mitochondria [32] an NADH dehydrogenase may be situated on the outer face of the inner mitochondrial membrane. Unlike yeast mitochondria [33], however, this NADH dehydrogenase in *Physarum* mitochondria is associated with a phosphorylation site, so that ADP/O values approaching 3, rather than 2, are obtained. The oxidation of exogenous or endogenous (pyruvate-malate generated) NADH is insensitive to rotenone. This insensitivity is unlikely to be due to impermeability of the mitochondria to the inhibitor, since as is shown below, rotenone has clear effects in relieving Ca^{2+} inhibition of respiration.

That the mitochondria have pathways of electron transport and coupled phosphorylation which are not greatly dissimilar to higher organisms is suggested by the data summarised in Table II, illustrating the effects of a range of inhibitors and uncouplers on respiration in *Physarum* mitochondria. Dinitrophenol at low concentration stimulates respiration to a significant extent, though not as much as ADP. At higher concentration dinitrophenol becomes inhibitory. In notable contrast to dinitrophenol, however, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton ionophore which uncouples oxidative phosphorylation in mammalian mitochondria, is without effect on *Physarum* mitochondria. Atractyloside, which inhibits adenine transport across the inner membrane of mammalian mitochondria, and oligomycin, both show a small but reproducible inhibition of respiration. The effects of these inhibitors (with the exception of CCCP), the stimulation of respiration by ADP, and the phosphorylation of ADP in significant quantities as shown by the ADP/O values, all indicate a substantial degree of coupling of phosphorylation to respiration in these mitochondria.

TABLE II

THE EFFECT OF ELECTRON TRANSPORT INHIBITORS AND UNCOUPLERS ON RESPIRATION IN *PHYSARUM* MITOCHONDRIA

The respiration rate of mitochondria with succinate as substrate has been arbitrarily set at 100. The actual respiratory activivity of the mitochondria used in this experiment was 160 nmol O_2/min per mg protein and the protein concentration in the assay was 0.4 mg/ml. Where ethanol was used as solvent for the inhibitor concerned, a correction was made for the small effects of ethanol alone.

Inhibitor	Respiratory activity
None	100
ADP, 140 μM	165
Dinitrophenol	
20 μM	131
250 μM	60
CCCP	
1 μM	100
10 μM	98
Rotenone	
0.5 μM	102
50 μM	101
Antimycin A	
0.5 μg/ml	100
20 μg/ml	59
Potassium cyanide	
1 mM	8
2 mM	2
Atractyloside	
50 μM (+ADP)	82
600 μM (+ADP)	68
Oligomycin, 20 μg/ml (+ADP) *	80

* Atractyloside or oligomycin were added in these assays immediately after stimulation by 140 μM ADP. The values should therefore be compared with those given for the ADP-stimulated rate.

That the electron transport chain may be unusual in certain respects is suggested by data obtained with respiratory inhibitors, also given in Table II. Each of the inhibitors was used over a range of concentrations which at its lower end is similar to that used for complete effect in mammalian preparations.

Rotenone, which, as mentioned earlier, has little effect on NADH oxidation at the highest concentrations tested is also without effect on succinate oxidation. Antimycin A inhibits succinate oxidation by less than half even at high concentrations. Although not shown here, the succinate : cytochrome *c* reductase activity of *Physarum* mitochondria was found to be insensitive to antimycin A. Cyanide is an effective inhibitor of succinate oxidation, though the oxidation of ascorbate/TMPD by *Physarum* mitochondria, in which electrons enter at the level of cytochrome *c* oxidase in the classical yeast and mammalian respiratory chain, is stimulated 30—40% by 1 mM potassium cyanide. Although not shown here, cyanide (1 mM) only partly inhibits (30 and 80%, respectively) respiration supported by pyruvate-malate and NADH, suggesting that a shunt mechanism may be operative, as has been described in mitochondria from other simple eukaryotes [26].

Examination of cytochrome spectra showed maxima at 601 (*a*-type), 548 (*c*-type) and 519 nm (*c*-type, β-band), with shoulders at 556 (*b*-type), 554 (c_1-type) and 526 nm (c_1-type, β-band). This pattern is similar to that shown by yeast mitochondria [26], except that the *a*-type cytochrome absorbance band in *Physarum* is at a slightly longer wavelength.

4. Respiratory response of mitochondria to Ca^{2+}

Coupled mitochondria respond to the addition of Ca^{2+}, with a 3—4-fold stimulation of their respiration (Fig. 2a, d). As with rat liver mitochondria the Ca^{2+}-stimulated rate is faster than the rate achieved in the presence of ADP, and the amount of extra oxygen consumed during the Ca^{2+}-stimulated respiration has a stoichiometric relationship to that induced during ADP phosphorylation [27]. This is clearly evident with the oxidation of NADH shown in Fig. 2c where the extent of the respiratory stimulation induced by 0.6 μmol Ca^{2+} is the same as that induced by 0.3 μmol ADP.

Stimulation of respiration is observed with as little as 50 μM Ca^{2+} (Fig. 3a). Above 200 μM Ca^{2+}, oxidation of trichloroacetic acid-cycle intermediates is inhibited after an initial, dampened respiratory stimulation in the presence or

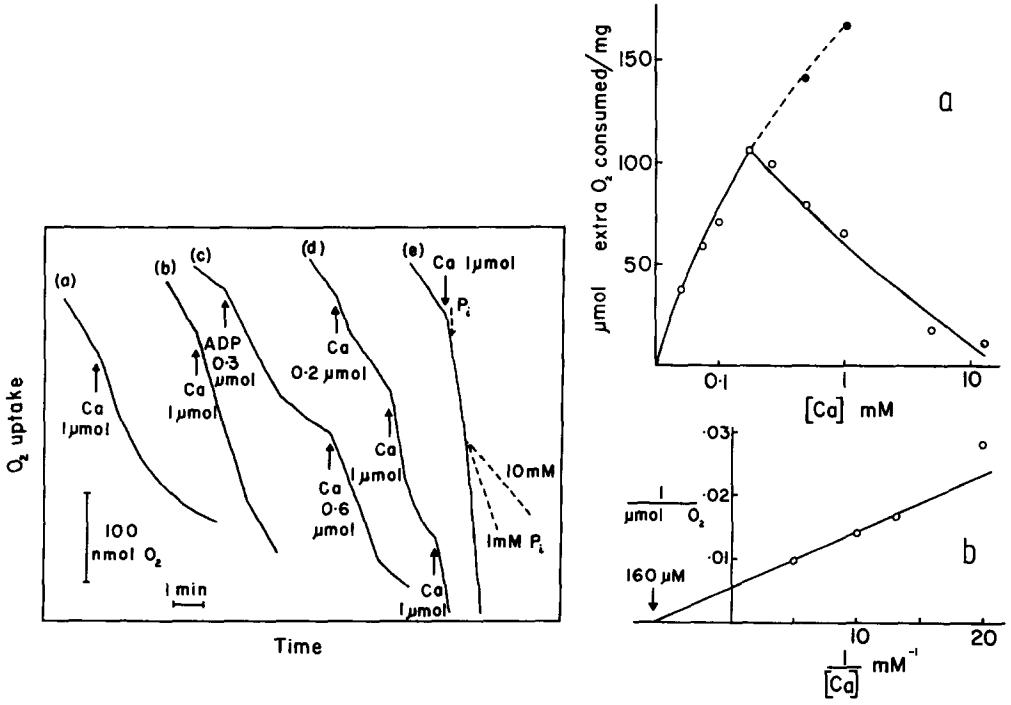

Fig. 2. The respiratory response of mitochondria to Ca^{2+}. In (a) and (b) mitochondria are oxidizing 5 mM succinate, and in (b) 10 μM rotenone is included. 1 mM NADH is the substrate in (c), (d) and (e). In (e) phosphate has been omitted from the respiration medium and the broken lines indicate the effect of adding phosphate to the incubation after the addition of Ca^{2+}.

Fig. 3. Extra O_2 consumed during Ca^{2+}-stimulated respiration. The extra O_2 consumed following the addition of Ca^{2+} was estimated as described by Rossi and Lehninger [27] in mitochondria oxidizing 5 mM succinate in the absence (○) and presence (●) of 10 μM rotenone. At Ca^{2+} concentrations of 200 μM and below, the addition of rotenone had no effect.

absence of phosphate. Reed and Bygrave [28] observed a similar response with rat liver mitochondria in a sucrose-based respiration buffer containing the permeant anion, CH_3COO^-. These responses seem to differ from the inhibited rate of mitochondria oxidation in the presence of Ca^{2+} in liver mitochondria (State 6) which is abolished by the permeant anion, PO_4^{3-} [34].

With succinate as substrate, the rate of oxygen uptake subsequent to stimulation by Ca^{2+} is slower than the prestimulated rate (Fig. 2a). Moreover, these succinate-supported mitochondria do not respond to further additions of Ca^{2+} nor of ADP, which is in contrast to NADH-supported mitochondria (Fig. 2d). This insensitivity to further addition of Ca^{2+} is observed in the presence and absence of phosphate. Mitochondria respiring on pyruvate-malate respond in a similar manner to those on succinate.

This effect has also been observed in rat liver mitochondria, where it is overcome by rotenone [28]. A similar relief of inhibition by rotenone is seen with *Physarum* mitochondria (Figs. 2b and 3a).

In rat liver mitochondria half the maximal extra oxygen consumption occurs at a concentration of 154 μM Ca^{2+} [27]. In the case of *Physarum* mitochondria, the corresponding value is 160 μM Ca^{2+} (Fig. 3b), demonstrating that by this measurement both *Physarum* and rat liver mitochondria respond similarly to Ca^{2+}.

Different respiratory responses were observed when Ca^{2+} was added to mitochondria respiring with NADH as substrate. In medium containing phosphate, a cyclic response to multiple additions of Ca^{2+} occurs (Fig. 2d). The block to further stimulation of oxygen uptake (seen when succinate is substrate (Fig. 2a)) is not seen even at Ca^{2+} concentrations as high as 2 mM. In medium lacking phosphate, low concentrations of Ca^{2+} induce an 'uncoupled' response (Fig. 2e). The addition of 10 mM phosphate early after the addition of Ca^{2+} prevents this, and restores the cyclic response. 1 mM phosphate is only partly effective in this respect.

The oxidation of external NADH appears to be coupled to Ca^{2+} uptake in a unique way. First, the block to further stimulation of respiration by Ca^{2+} when succinate or pyruvate-malate are respiratory substrates, discussed above, is not apparent with NADH provided phosphate is present (Fig. 2d). Second, in the absence of phosphate, the addition of Ca^{2+} to mitochondria oxidizing NADH, produces a response similar to that of inhibitors which uncouple oxidation from phosphorylation and produce a continuously stimulated respiration rate (Fig. 2e). The reasons for this unusual effect are not known. The fact that phosphate added after this response has been induced restores the apparent coupling between Ca^{2+} uptake and oxygen consumption (Fig. 2e) demonstrates that the effect is reversible. La^{3+} has a similar effect as phosphate, but mitochondria are then insensitive to further additions of Ca^{2+}. It is feasible that there is a relationship between the oxidation of exogenous NADH (perhaps involving an NADH dehydrogenase located on the outer surface of the inner mitochondrial membrane as in yeast [32]), the uptake of Ca^{2+} and phosphate, and the unusual formation of Ca^{2+} deposits in the space between the inner and outer mitochondrial membranes observed by Nicholls [36].

Sr^{2+} (0.5 mM) is equally as effective as Ca^{2+} in stimulating respiration in medium lacking phosphate. Mn^{2+} (0.5 mM) stimulates respiration only slightly, and Ba^{2+} (0.5 mM) not at all.

NADPH is oxidized at a rate similar to NADH, and the addition of Ca^{2+} produces the same effects. NAD has no effect on respiration rates.

5. $^{45}Ca^{2+}$ uptake by Physarum mitochondria

Initial experiments on measurements of $^{45}Ca^{2+}$ uptake were sometimes hampered by a tendency of the mitochondria to clump in the assay buffers. However, provided large sample volumes are used, the variation consequent upon this clumping phenomenon can be overcome. Ca^{2+} uptake shows rapid initial rates, with uptake being essentially complete within 1 min at 25°C (Fig. 4). With a Ca^{2+} concentration of 0.1 mM in the medium, mitochondria accumulated 120 nmol Ca^{2+}/mg protein, which represents almost 80% of the Ca^{2+} in the medium. Uptake is very much reduced at 0°C (Fig. 4).

When the Ca^{2+} dependence of the initial velocity of Ca^{2+} uptake in nitrilotriacetate-buffered medium is examined, a sigmoidal relationship between initial velocity and concentration is observed (Fig. 5), suggesting that there is a cooperative effect between Ca^{2+} and Ca^{2+}-transport similar to that which occurs in rat liver mitochondria [9]. Half maximal velocity of Ca^{2+} uptake occurs at approx. 50 μM Ca^{2+} which is about 10-fold greater than the K_m recently determined for mammalian mitochondria [9]. The maximal velocity of Ca^{2+} uptake attained with *Physarum* mitochondria at 25°C (approx. 15 nmol/s per mg) is similar to that detected in mammalian mitochondria (8—14 nmol/s per mg) [30].

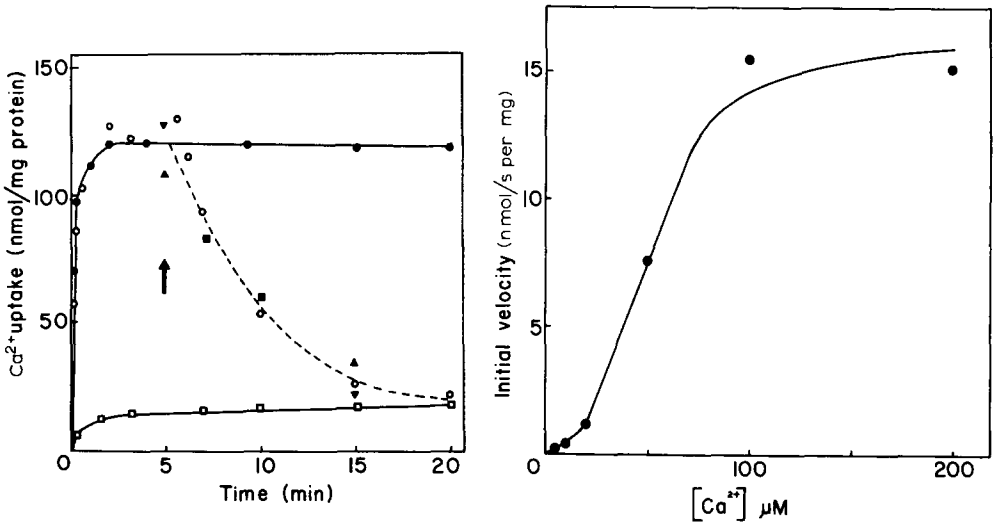

Fig. 4. $^{45}Ca^{2+}$ uptake by mitochondria and the effect of KCN, EGTA, La^{3+} and ruthenium red on $^{45}Ca^{2+}$ efflux. $^{45}CaCl_2$ was added to the basic Ca^{2+} uptake medium containing mitochondria (0.65 mg/ml) to give a final concentration of 0.1 mM. Additions made after incubation for 5 min were: 1 mM EGTA (○), 500 μM La^{3+} (▼), 4 μM ruthenium red (▲), 1 mM KCN (■), and none (●). $^{45}Ca^{2+}$ uptake at 0°C was also monitored (□).

Fig. 5. The effect of Ca^{2+} concentration on the initial velocity of mitochondria $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake was measured by withdrawing a 0.1 ml sample from 0.2 ml assay medium containing 20 mM nitrilotriacetate and 0.08 mg of mitochondrial protein after 10 s incubation. Nitrilotriacetate Ca^{2+} buffers [29] were used to generate known concentrations of free Ca^{2+}. Nitrilotriacetate had no effect on mitochondrial respiration. The points represent the means of two experiments.

Endogenous respiration supported the uptake in 5 min of 50% of the Ca^{2+} uptake obtained in the presence of succinate, whereas with ATP as energy source 85% was incorporated. The free Ca^{2+} was computed in the presence of ATP and in succinate medium where the Ca^{2+} was buffered with nitrilotriacetate. 1 mM KCN present before Ca^{2+} was added results in an approx. 90% inhibition of Ca^{2+} uptake. The addition of KCN to mitochondria pre-loaded with $^{45}Ca^{2+}$ induces the efflux of 80% of the $^{45}Ca^{2+}$ in 5 min, and a similar efflux is observed when 1 mM EGTA is added (Fig. 4).

La^{3+} and ruthenium red, inhibitors of Ca^{2+} uptake by mammalian mitochondria [37,38], are also potent inhibitors of Ca^{2+} uptake by *Physarum* mitochondria. Half maximal inhibition of uptake is observed with 10 μM La^{3+} and 1 μM ruthenium red (Fig. 6). Both inhibitors induce an efflux of $^{45}Ca^{2+}$ from preloaded mitochondria similar to that caused by KCN and EGTA. These results suggest that in *Physarum* mitochondria Ca^{2+} uptake is an active process whereas efflux is passive and that efflux may not occur through the Ca^{2+} carrier.

Results concerning the effect of La^{3+} and ruthenium red on Ca^{2+} efflux from mitochondria have been contradictory [39] and the mechanism of Ca^{2+} efflux is still not certain. Hence caution must be used in experiments aiming to determine the compartmentation of Ca^{2+} within a cell type to ensure that substantial Ca^{2+} efflux does not occur. La^{3+} [40] and ruthenium red [41] have been used to prevent Ca^{2+} redistribution in such studies in liver and adipose tissue, but our studies indicate that substantial efflux of Ca^{2+} from *Physarum* mitochondria would be promoted under these conditions.

The isolation conditions reported here will enable Ca^{2+} uptake to be studied in detail to determine the physiological role of *Physarum* mitochondria in controlling cytoplasmic Ca^{2+} levels. With further characterization of the Ca^{2+}-transporting microsomal fraction recently reported in *Physarum* [12], it should soon be possible to achieve a more complete understanding of the regulation of Ca^{2+} in the cellular compartments of this organism.

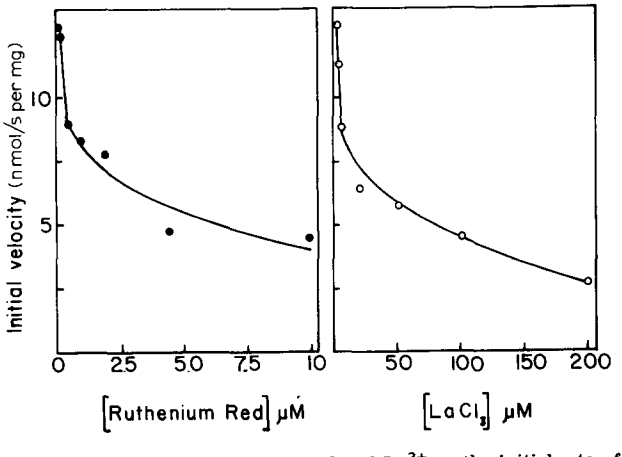

Fig. 6. The effect of ruthenium red and La^{3+} on the initial rate of Ca^{2+} uptake. Inhibitors were added 30 s before 100 μM $^{45}CaCl_2$ (final concentration) was added to the assay medium containing 0.5 mg protein/ml. The uptake in 10 s was estimated.

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