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The Role of Mitochondria in Modifying the Cellular Ionic Environment: Studies of the Kinetic Accumulation of Calcium by Rat Liver Mitochondria

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Abstract

1. The affinity of ATP-supported Ca^{2+} accumulation for both Ca^{2+} and ATP was determined from initial rate studies employing isolated rat liver mitochondria. The K_m values for "free" Ca^{2+} and ATP were calculated to be of the order of 2 μM and 100 μM, respectively. The K_m for ATP decreased as the Ca^{2+} concentration was increased.

2. The curve relating initial rates of Ca^{2+} accumulation to Ca^{2+} concentration was sigmoidal in shape; values obtained for the Hill coefficient were in the range 1.5-1.9.

3. Concomitant with the ATP-stimulated accumulation of Ca^{2+}, ATP translocation was itself increased in the presence of Ca^{2+}. This stimulation took place independently of Ca^{2+} accumulation.

4. Decreasing the pH of the incubation medium decreased the rate of Ca^{2+} accumulation. This inhibition was competitive in that the affinity of mitochondria for Ca^{2+} could be altered. The maximal rate of accumulation did not change with change in pH.

5. The permeant anions inorganic phosphate and acetate stimulated the accumulation of Ca^{2+} in a non-competitive manner. Both the V_{max} and K_m varied when either of the anions were present.

6. The data are discussed in relation to the role that mitochondria play in controlling the cellular ionic environment.

Introduction

Over the last decade considerable insight has been gained in the mechanism of Ca^{2+} accumulation by isolated mitochondria. Despite this

Non-usual abbreviations:

K_m, the substrate concentration at ½ V_{max}; n, the Hill coefficient, is the negative slope of a plot of log V_{max}/v-1 versus log s, and substrate concentration at velocity v; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid.

knowledge, however, it is still far from clear what relation this process bears to the overall cellular metabolic network. Lehninger [1] and ourselves [2-4] have previously expressed the view that ion accumulation by mitochondria is involved in controlling the ionic environment in the cell.

If Ca^{2+} accumulation by mitochondria is to have an effective role in modifying the internal cellular ionic environment, it might be expected to have a high affinity not only for Ca^{2+} but also for the metabolite supporting the active accumulation of the ion. Because of this expectation we have made a detailed *kinetic* analysis of Ca^{2+} accumulation so that precise data can be made available on the affinity of the process for Ca^{2+} and for the metabolite providing the energy for accumulation.

Results from our experiments provide, for the first time, definitive K_m values both for Ca^{2+} and for ATP, the source of energy in these experiments. The magnitude of these values indicates that the affinity of ATP-supported Ca^{2+} accumulation both for Ca^{2+} and for ATP is high and that the value for each is dependent on the concentration of the other in the medium. These findings are interpreted as supporting the view that mitochondria play an essential role in controlling the intracellular ionic environment, a further means whereby mitochondria modify cell metabolism.

Methods and Materials

Preparation of Mitochondria

Mitochondria were prepared from the livers of 200 g male Wistar strain albino rats essentially by the method of Schneider [5]. The preparation was carried out in a lightly buffered 0.25 M sucrose solution containing 2 mM HEPES-KOH, pH 7.4. Two washes of the mitochondria were usually employed. All steps were carried out at approximately 4° with the final resuspension being made in isolation medium at a concentration of 25-30 mg of mitochondrial protein per ml.

Conditions of Incubation

All experiments were performed with a basic incubation medium of 200 mM sucrose and 2 mM HEPES-KOH, pH 7.4. One to 1.2 mg of mitochondrial protein/ml were usually present. Other additions were made as indicated in the legends to the figures and tables. The incubation vessel consisted of a small water-jacketed glass container, the medium being mixed by the action of a small magnet. Unless otherwise indicated the temperature of this vessel was thermostatically controlled at 25°.

Mitochondria were preincubated in the reaction media for 1 min to obtain temperature equilibration. The reaction was then started by the addition of appropriate amounts of $^{45}CaCl_2$ diluted with non-radioactive $CaCl_2$.

Samples and Counting

Ten seconds after the addition of the radioactive calcium, 100 μl of the incubation mixture was removed and filtered by means of a millipore filtration device using either Gelman GN-6 or Millipore filters of 0.45 μ pore size. Pre-filtering and post-filtering washings with 2.5 ml of basic incubation medium were used. The filter was then placed in a glass vial, dried and 10 ml of liquid scintillation fluid containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis(2-)5-phenoloxazolyl benzene made up in 1 litre of toluene, were added. Radioactivity was counted by means of a packard Tricarb Scintillation Counter to 1% error on the ^{14}C-channel.

Specific Activity

Specific activities were determined by removing a 100 μl sample of the incubation medium, placing it on a filter paper in a glass vial, drying and counting as above.

Protein Estimation

Protein was assayed according to a biuret method. The mitochondria were treated with 2% deoxycholate to solubilize the mitochondrial membranes.

Presentation of Data

Although the duration of each experiment was only 10 sec the results have been recalculated and except where indicated expressed as nanomoles of Ca^{2+} accumulated/mg of mitochondrial protein/min. Some data are expressed in terms of "free" Ca^{2+}; these values were determined from the concentration of added Ca^{2+} and added ATP corrected for the stability of the complexes $CaATP^{2-}$ and $CaHATP^{1-}$ which these two species form [6].

Concentration of Inhibitors Used

Metabolic inhibitors when present were used at concentrations which control experiments indicated were sufficient to maximally inhibit oxidative phosphorylation. Antimycin A was used at 0.225 μM, rotenone

at 0.25 μM, oligomycin at 2.5 μg/ml, atractyloside at 5 μM and DNP at 0.1 mM.

Materials

$^{45}CaCl_2$ (0.507 mC/mM) and 8-^{14}(C)ATP (50 μC/ml) were obtained from The Radiochemical Centre, Amersham, England. ATP was obtained from Boehringer and Soehne, Mannheim, Germany. Antimycin A, rotenone and oligomycin were obtained from the Sigma Chemical Co. Atractyloside was a gift from Dr. E. Carafoli. All other reagents were of A.R. grade.

Results

In most studies, Ca^{2+} accumulation by isolated mitochondria has been examined using a respiration-supported system. Those experiments which have been carried out in an ATP-supported system have involved thermodynamic measurements of Ca^{2+} accumulation [7-11]. Thus from these studies it has not been possible to obtain definitive information on the affinity of ATP-supported Ca^{2+} accumulation either for ATP or for Ca^{2+}.

Data in Fig. 1 show the amount of Ca^{2+} accumulation by isolated rat liver mitochondria in the presence of two different concentrations of ATP. After 10 sec Ca^{2+} accumulation is far from complete in each medium; in the presence of 6 mM ATP only 40% of the Ca^{2+} available has been accumulated while in the presence of 1.2 mM ATP only about 60% has been accumulated. Accumulation approaches completeness only after 1 and 2 min in the presence of the lower and higher ATP concentrations, respectively. These data indicate that sampling after a 10 sec incubation period reflects a kinetic measurement of Ca^{2+} accumulation in the present system.

ATP-Supported Ca^{2+} Accumulation Examined as a Function of Ca^{2+} Concentration

Figure 2 shows the influence of Ca^{2+} concentration on the rate of Ca^{2+} accumulation supported by ATP. The curve relating initial rate of accumulation to Ca^{2+} concentration is non-linear both with and without antimycin A and rotenone. The presence of the inhibitors, however, decreases the extent of Ca^{2+} accumulation. This inhibition seems to be non-competitive in that the apparent affinity for Ca^{2+} does not change significantly. After correction for complexing with ATP (see Methods) the K_m was calculated to increase slightly from 1.5 μM in the non-

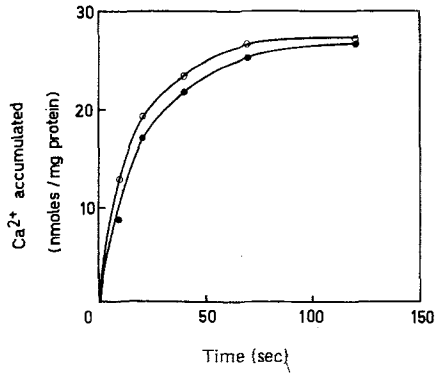

Figure 1. Time course for ATP-supported Ca^{2+} accumulation. Mitochondria were incubated as indicated in Methods in the basic medium supplemented with 80 μM Ca^{2+} (containing $^{45}Ca^{2+}$), 2 mg mitochondrial protein and ATP as indicated for the times shown. The final volume was 1.0 ml. ●, 6 mM ATP; ○, 1.2 mM ATP.

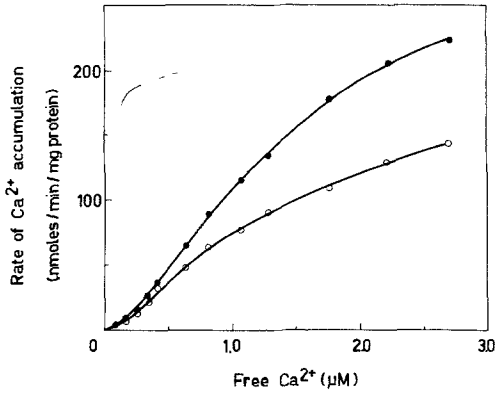

Figure 2. Effect of Ca^{2+} concentration on initial rate of ATP-supported Ca^{2+} accumulation. Mitochondria were incubated as indicated in Methods in the basic medium supplemented with 6 mM ATP and Ca^{2+} as indicated. Incubations were for 10 sec at 25°. ●, no inhibitors present; ○, antimycin A and rotenone present.

inhibited system to 1.6 μM in the inhibited. The V_{max} decreased from 313 to 182 nmoles Ca^{2+} accumulated per mg protein per min in the presence of antimycin A and rotenone while the Hill coefficient n a measure of the co-operativity of the system [12], decreased from 1.6 to 1.5.

Effect of 6 mM ATP on Succinate-supported Ca^{2+} Accumulation as a Function of Ca^{2+} Concentration

As a comparison with ATP-supported accumulation, the effect of Ca^{2+} concentration on succinate-supported accumulation was investigated (Fig. 3). The hyperbolic-shaped plot obtained in the absence of added

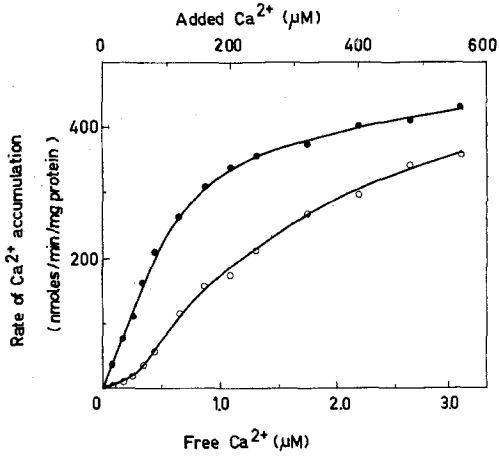

Figure 3. Effect of 6 mM ATP on succinate-supported Ca^{2+} accumulation as a function of Ca^{2+} concentration. Mitochondria were incubated as indicated in Methods in the basic medium supplemented with 2 mM succinate, oligomycin and Ca^{2+} as indicated. Incubations were for 10 sec at 25°. ●, no ATP present; ○, 6 mM ATP present. The "free" Ca^{2+} values refer only to the data obtained in the presence of ATP. The "added" Ca^{2+} values refer to both sets of data.

ATP does not reflect true Michaelis-Menten kinetics because *complete* accumulation of Ca^{2+} (up to 80 μM) from the suspending medium has taken place. On the other hand the addition of 6 mM ATP together with oligomycin (to prevent its hydrolysis), allowed initial rates of accumulation to be measured. Under these circumstances, a sigmoid-shaped saturation curve was obtained. Upon correction for complexing of Ca^{2+} with ATP (see Methods), the apparent affinity constant of the system for Ca^{2+} was calculated to be 1.8 μM. This compares favourably with the K_m obtained for Ca^{2+} in the ATP-supported system under similar conditions (see above). The Hill coefficient for the succinate-supported system was 1.75 and the V_{max} approximately 450 nmoles Ca^{2+} accumulated per mg protein per min. It should be noted that in the presence of 6 mM ATP, the "free" Ca^{2+} concentration was decreased approximately 200-fold in the range of Ca^{2+} concentrations examined.

Effect of ATP Concentration on ATP-Supported Ca^{2+} Accumulation

Data in Fig. 4 show the effect of various metabolic inhibitors on Ca^{2+} accumulation as a function of ATP concentration. In the absence of inhibitors, i.e. conditions which allow both binding and accumulation to occur, a slight stimulation is evident until about 20 μM ATP is reached.

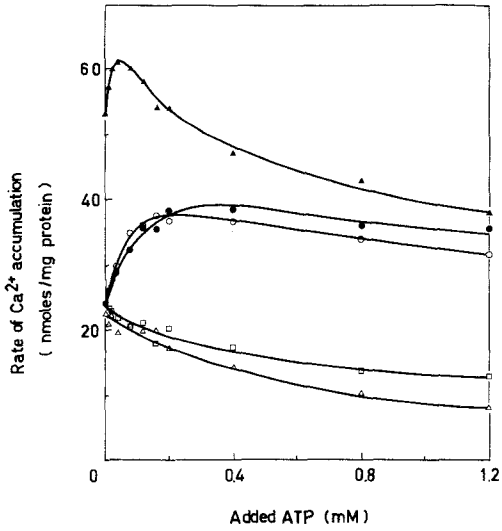

Figure 4. Effect of ATP concentration on ATP-supported Ca^{2+} accumulation. Mitochondria were incubated as described in Methods in a basic medium supplemented with ATP, as indicated, and 240 μM Ca^{2+}. Incubations were for 10 sec at 25°. ▲, no inhibitors present; ●, antimycin A and rotenone present; ○, antimycin A and rotenone present (no preincubation of ATP, see notes); □, antimycin A, rotenone, atractyloside ± oligomycin present; △, DNP present.

The extent of binding/accumulation begins to decline as the ATP concentration is increased further. In the presence of antimycin A and rotenone stimulation of Ca^{2+} accumulation was also observed on the addition of ATP. The stimulation was seen up to a concentration of about 300 μM ATP. Control experiments indicated that initial rates of Ca^{2+} accumulation were being measured also at these concentrations of ATP. When atractyloside or DNP were present, no stimulation of Ca^{2+} accumulation by ATP was observed. The amount of Ca^{2+} accumulated in the presence of antimycin A and rotenone and atractyloside was taken as the amount of Ca^{2+} "bound" to the mitochondria at both the low and high affinity binding sites (see ref. 13). By subtracting the amount of Ca^{2+} "accumulated" it was possible to obtain the actual amount of Ca^{2+}

"entering" the mitochondria. Thus results in Fig. 5, calculated from those shown in Fig. 4 show the amount of Ca^{2+} entering the mitochondria as a function of ATP concentration. In the presence of DNP, which has been shown to inhibit both the uptake and the high affinity binding of Ca^{2+} by mitochondria [13], the amount of Ca^{2+} associated with the mitochondria was decreased over the range of ATP concentrations studied (0-1.2 mM) to values similar to those obtained in the presence of atractyloside.

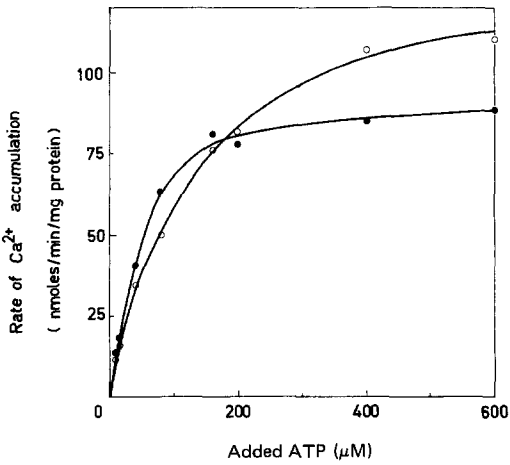

Figure 5. Effect of ATP concentration on actual Ca^{2+} accumulation. The data have been recalculated from Fig. 4. ○, antimycin A and rotenone present; antimycin A and rotenone present (no preincubation of ATP).

Slight differences were noted in the presence of antimycin A and rotenone when the required ATP was added simultaneously with the Ca^{2+}. The degree of stimulation was increased at low concentrations and the ATP concentration at which inhibition of accumulation was first observed decreased to about 150 μM. After correction for binding, as shown in Fig. 5, the degree of stimulation due to ATP fell by about 15% at the higher concentrations of ATP. These differences, obtained when ATP was added at the same time as Ca^{2+}, were most probably caused by the translocation of ATP in the 1 min interval before the addition of Ca^{2+}. In the following experiments, except where indicated, Ca^{2+} and ATP were added simultaneously to the system to minimize effects due to translocation of ATP.

Stimulation of ATP-Supported Ca^{2+} Accumulation by ATP in the Presence of Various Ca^{2+} Concentrations

Table I shows the effect of various added Ca^{2+} concentrations on the kinetic constants of ATP-stimulated and -supported accumulation of Ca^{2+}. The additions of Ca^{2+} and ATP to the incubation medium were made at the same time to minimize the effects caused by the translocation and subsequent lowering of the concentration of the added ATP. An inverse relationship between V_{max} and K_m was observed. The V_{max} increased from 35 to 112 nmoles Ca^{2+} accumulated per mg protein per min when the added Ca^{2+} concentration was increased from 24 to 240 μM. The K_m values for ATP varied from 96 μM at 24 μM Ca^{2+} to 53 μM at 240 μM added Ca^{2+}. It should be noted that values of less than 1 for the Hill coefficient were obtained in these experiments.

TABLE I. Kinetic constants of ATP-supported and stimulated Ca^{2+} accumulation in the present of various added Ca^{2+} concentrations

Ca^{2+} μM	V_{max}	K_m	n
24	35	96	1.0
80	63	60	0.53
120	73	55	0.70
160	85	55	0.64
240	112	53	0.80

Mitochondria were incubated, as in Methods, in the basic medium supplemented with antimycin A, rotenone, varying ATP concentrations and Ca^{2+} as indicated. ATP and Ca^{2+} were added together. After correction for binding (see Figs. 4 and 5) V_{max} values were determined using a Lineweaver-Burk plot. From the V_{max} values the K_m and the Hill coefficient, n, were calculated using the appropriate Hill plot.

ATP Accumulation by Mitochondria

It was of interest to determine what effect (if any) Ca^{2+} was having on the translocation of ATP measured as a function of ATP concentration. Data in Fig. 6 show the effect of increasing ATP concentration on the exchange of ATP in the presence of and absence of Ca^{2+} and metabolic inhibitors. The experiments revealed several interesting points. Firstly, Ca^{2+} greatly stimulates the translocation of ATP. Secondly, in the absence of Ca^{2+} the curve did not appear to approach an asymptote in the ATP concentration range. When Ca^{2+} was present a distinct saturation point was observed. It must be emphasized that the results presented here do not represent initial velocity kinetics (see ref. 14). Thirdly, the addition of oligomycin to the incubation medium did not diminish the stimulatory effect of Ca^{2+} on the translocase activity.

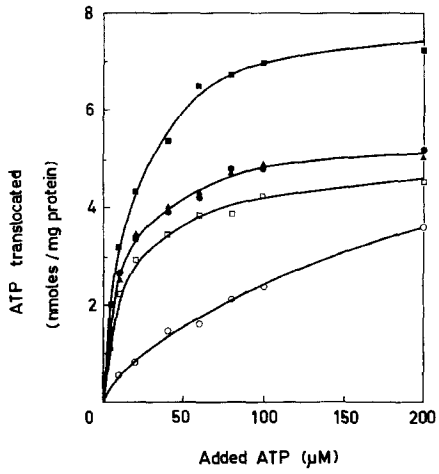

Figure 6. Effect of ATP concentration on ATP translocation in the presence and absence of 240 μM Ca^{2+}. Mitochondria were preincubated as described in Methods in a basic medium supplemented with antimycin A and rotenone. ^{14}C-ATP was added, as indicated, synchronously with Ca^{2+}. The nmoles of ATP translocated represent the ATP entering the mitochondria after a 10 sec incubation period. The temperature was 25°. ▲, 240 μM Ca^{2+} present; ●, oligomycin and 240 μM Ca^{2+} present; ■, DNP and 240 μM Ca^{2+} present; ○, no Ca^{2+} present; □, DNP present.

Fourthly, the uncoupler-stimulated exchange of ATP [15] and Ca^{2+}-stimulated exchange seem to be additive. More detailed studies on Ca^{2+} stimulation of adenine nucleotide exchange are considered by us elsewhere [16-18].

Effect of pH on the Initial Rate of Ca^{2+} Accumulation

Table II shows the effect of pH on the kinetic constants obtained from experiments in which Ca^{2+} accumulation as a function of Ca^{2+} concentration was compared at four different pH values. The V_{max} values do not vary from the value of 250 nmoles accumulated per min per mg protein. The affinity of the system for Ca^{2+} increases as the pH increases. Between pH 6.9 and 8.4 the K_m drops by about 50% from 2.3 to 1.2 μM "free" Ca^{2+}. Similar trends to those obtained here were observed by Reynafarje and Lehninger [13] and Scarpa and Azzi [19] for the effect of pH on the affinity of low affinity Ca^{2+} binding and by Reynafarje and Lehninger [13] on the high affinity binding. However these latter authors also observed a concomitant increase in the number of low affinity binding sites (which may be correlated with V_{max}) from 30 nmoles/mg protein at pH 6.7 to about 50 nmoles of Ca^{2+} bound at

TABLE II. Effect of pH on kinetic constants of ATP-supported Ca^{2+} accumulation

pH	V_{max} nmoles Ca^{2+} accumulated/mg protein/minute	K_m μM "free" Ca^{2+}	n
6.9	250	2.3	1.8
7.4	250	2.0	1.9
7.9	250	1.6	1.9
8 4	250	1.2	1.8

Conditions as in Methods. V_{max} values were determined using a Lineweaver-Burk plot. From the V_{max} values the K_m and Hill coefficient, n, were calculated using the appropriate Hill plot.

pH 9.0. The Hill coefficient remains constant and is not pH-dependent over the pH range studied.

Effect of Permeant Anions on Initial Rate of Ca^{2+} Accumulation

1. Effect of anion concentration on Ca^{2+} accumulation. Permeant anions are known to promote the accumulation of Ca^{2+} by isolated mitochondria [20-22]. Data in Fig. 7 show that in both the ATP- and succinate-supported systems, phosphate and acetate also stimulate the *initial* rate of Ca^{2+} accumulation by rat liver mitochondria. As a function of anion concentration the accumulation shows a Michaelis-Menten relationship. The overall rates of accumulation are lower in the succinate-supported system than in the ATP-supported system at the anion concentrations tested. The degree of stimulation is greater at the higher concentrations of Ca^{2+}. Over the phosphate range tested, a stimulation of about 60% is observed at 240 μM added Ca^{2+}, i.e. 2.71 μM "free" Ca^{2+}. Under all conditions half-maximal stimulation occurs at about 0.5 mM phosphate and 0.7 mM acetate.

2. Effect of anions on Ca^{2+} accumulation as a function of Ca^{2+} concentration. Data accumulated in Table III show the effect of phosphate and of acetate on the kinetic constants in both ATP-supported and succinate-supported systems. Under all conditions the value of the Hill coefficient is 1.9. In the succinate-supported system in the presence and absence of 1.0 mM phosphate and in the ATP-supported system in the absence of phosphate the apparent K_m is of the order of 1.95 μM "free" Ca^{2+}. However, in the ATP-supported system the K_m increased to 2.3 μM in the presence of 1 mM phosphate. The presence of phosphate increases the V_{max} by about 65%, from 450 to 770 nmoles/min/mg protein in the ATP-supported system and from 350 to 520 in the succinate-supported system. The observed stimulatory

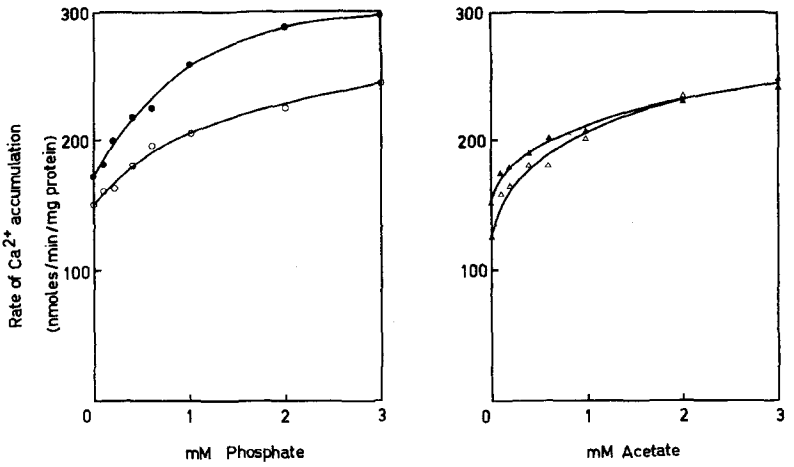

Figure 7. Effect of anion concentration on initial rate of Ca^{2+} accumulation. Mitochondria were incubated in a basic medium supplemented with 6 mM ATP plus antimycin A (closed symbols) or 2 mM succinate plus oligomycin (open symbols) and anions (as the potassium salt) at the concentrations indicated. 240 μM Ca^{++} was initially present. Rotenone was added to all incubation mixtures.

effects of phosphate on Ca^{2+} accumulation are in agreement with the results obtained by Haugaard *et al.*, [10].

The addition of 1 mM acetate to the incubation medium stimulates the accumulation of Ca^{2+} over the entire Ca^{2+} concentration range. Table III shows that the presence of 1 mM acetate increased the V_{max} of the succinate-supported system from 350 to 530 nmoles Ca^{2+} accumu-

TABLE III. Effect of anions, in both ATP- and succinate-supported systems, on kinetic constants of Ca^{2+} accumulation

	ATP-supported			Succinate-supported		
	V_{max}[a]	K_m[b]	n	V_{max}	K	n
Control	450	2.0.	1.9	350	2.0	1.9
Phosphate	770	2.3	1.9	520	2.0	1.9
Acetate	580	2.0	1.9	530	2.0	1.9

[a] nmoles Ca^{++} accumulated/min/mg protein.
[b] μM "free" Ca^{++}.

Conditions were in Methods. Antimycin A and rotenone were present in the ATP-supported systems, oligomycin and rotenone were present in the succinate-supported systems. Anions were present at 1 mM as the potassium salt. V_{max} values were determined using a Lineweaver-Burk plot. From the V_{max} values the K_m and Hill coefficient, n, were calculated using the appropriate Hill plot.

lated per min per mg protein. This compares with an increase from 450 to 580 in the ATP-supported system. Thus acetate stimulates the succinate-supported accumulation to a greater extent than that observed in the ATP-supported systems (50% stimulation compared with 30%). In all cases the K_m and Hill coefficient for the accumulation curves do not vary.

Discussion

Data obtained in the present work focus on previously neglected aspects of Ca^{2+} accumulation by isolated rat liver mitochondria. However, we believe these aspects of the mechanism of Ca^{2+} accumulation bear directly on the potential role of mitochondria in controlling the ionic environment in the cell. The first aspect is the high affinity of the ATP-supported accumulation both for Ca^{2+} and for ATP and the second the shape of the curve relating initial rates of Ca^{2+} accumulation to Ca^{2+} concentration.

The functions of ATP in the present experiments were two-fold, the particular function being controlled by the judicious use of metabolic inhibitors (see Figs. 2 and 3). In some experiments ATP served a dual function, that of an energy source and, by virtue of its strong ability to chelate Ca^{2+}, that of creating a Ca^{2+} "sink". In the succinate-supported systems, ATP served only the latter function and thereby enabled very low concentrations of "free" Ca^{2+} to be presented to the mitochondria at any one time. These circumstances permitted the kinetic measurements of Ca^{2+} accumulation (Fig. 1, refs. 23-25).

The very high affinity of the accumulation process for "free" Ca^{2+} was clearly indicated by these experiments. In each of many experiments, calculated values in the range 1-2 μM were consistently obtained [24, 25]. Carafoli [26] has recently reported (without presenting data) similar values. These affinity values for Ca^{2+} accumulation can be compared to those of about 0.4-1.6 μM obtained for high affinity binding of Ca^{2+} to rat liver mitochondria [13, 28]. The close agreement between these independent sets of data would favourably argue that we are making a significant kinetic measurement with the experimental techniques adopted. Other arguments supporting this contention are described elsewhere [27]. Although it is quite possible that ATP could have been exerting effects other than those indicated above, similar sigmoidal behaviour may also be observed in the absence of ATP and in succinate-supported system [25, 27]. This question is considered in detail elsewhere [27].

The V_{max} values obtained in the present work calculated from initial velocity data, ranged from about 100-400 nmoles/min/mg protein depending on the concentration and source of energy. These values are in

the range obtained by Mela and Chance [29]. On the basis of Ca^{2+} accumulation studies during state 4 respiration, Drahota *et al.* [30] put a lower limit to the rate of Ca^{2+} accumulation by rat liver mitochondria at about 40 nmoles/min/mg protein.

The relatively high affinity of ATP-supported accumulation for ATP is also indicated by data presented in this paper (see Table I). These experiments showed that K_m values for *total* ATP as low as 50 μM could be obtained. Bielawski and Lehninger [9] observed that a concentration of 0.5 mM ATP was required to obtain approximately optimal (non-kinetic) accumulation of Ca^{2+}. It was of further interest in the present work to find that the K_m for ATP varied according to the concentration of CA^{2+} presented to the mitochondria. Thus at low concentrations of added Ca^{2+}, i.e. at about 25 μM, where the V_{max} was least, the K_m for ATP was highest. At higher concentrations of Ca^{2+}, i.e. at about 120 μM, where the V_{max} was greatest, the K_m was least.

Data obtained in the present paper confirm and amplify our previous reports [23-25] that when initial rates of Ca^{2+} accumulation are determined as a function of Ca^{2+} concentration, non-hyperbolic saturation curves are obtained. In all of the experiments described, the Hill coefficient [12], which provides some idea of the "co-operativity" of the system, was of the order of 1.5-1.9. Although there is little supporting evidence, it is suggested that the accumulation of one Ca^{2+} ion facilitates the accumulation of a second. It is known that 1 ATP molecule (or hypothetical high-energy intermediate) is required for the accumulation of 2 Ca^{2+} ions [31].

It is now profitable to enumerate those features of the ion accumulation mechanism which support the proposition that mitochondria play a physiological role in controlling the ionic environment within the cell.

In the first place we have shown that the bivalent ion accumulation properties of rat liver mitochondria can be used to manipulate the Mg^{2+}/Ca^{2+} ratio in the cytosol [4] and thereby allow for modification of cytoplasmic metabolic activities, such as glycolysis [4, 32, 33], protein biosynthesis [34] and phospholipid biosynthesis [35], which are sensitive to this ratio. Secondly, we have shown that, provided care is taken in making kinetic measurements, the accumulation of Ca^{2+} by rat liver mitochondria when determined as a function of Ca^{2+} concentration exhibits not a hyperbolic but a sigmoidal curve [23-25, 27]. This feature, which is reminiscent of regulatory behaviour and consistent with the first point above, suggests that there is an upper limit to the concentration of Ca^{2+} which the cell will tolerate in its cytosol. When the concentration of Ca^{2+} becomes greater, the mitochondria are geared to accumulate Ca^{2+} in a "co-operative" manner. Thirdly, the present work has shown that ATP-supported Ca^{2+} accumulation has a very high

affinity both for "free" Ca^{2+} and "free" ATP. This observation, together with the finding that the affinity values for each of the two metabolites depends on the concentration of the other in the medium, ensures that a continual and efficient removal of Ca^{2+} from the cytosol into the mitochondria can take place irrespective of the ATP concentration outside the mitochondria.

Finally, we envisage that the Ca^{2+}-stimulated translocation of ATP [16, 17], which we believe constitutes a mechanism facilitating access of ATP to mitochondrial ATPase [17], is also geared to the need to modify the ionic environment in the cell. Further work designed to elaborate and test these propositions is in progress in our laboratory.

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