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THE FUNCTION OF ATP IN Ca^{2+} UPTAKE BY RAT BRAIN MITOCHONDRIA

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SUMMARY

Ca^{2+} uptake by rat brain mitochondria was studied under different experimental conditions. The most rapid uptake of Ca^{2+} occurred in the presence of ATP, succinate and P_i. ATP alone also supported Ca^{2+} uptake. In contrast, no Ca^{2+} uptake occurred with succinate and P_i when no ATP was added. Oligomycin and atractylate completely inhibited ATP-supported Ca^{2+} uptake but produced only a partial inhibition of Ca^{2+} transport in the presence of ATP, succinate and P_i. ATP plays a dual role in its action on brain mitochondria; it can support Ca^{2+} uptake by itself and it serves a function in allowing respiration-dependent Ca^{2+} uptake to proceed. The latter role of ATP does not involve transfer of energy from the nucleotide.

INTRODUCTION

The uptake of Ca^{2+} by mitochondria has been found to be a process requiring energy supplied by ATP or by respiration[1]. Liver mitochondria accumulate Ca^{2+} in the absence of added adenine nucleotides when a respiratory substrate, such as glutamate or succinate, is present. This process is markedly stimulated by addition of P_i (ref. 2).

In the experiments to be presented the effects of ATP and P_i on the uptake of Ca^{2+} by mitochondria from rat brain were studied. In contrast to liver mitochondria, mitochondria from brain were found to be unable to take up Ca^{2+} in the absence of ATP. With succinate as substrate no Ca^{2+} uptake occurred unless ATP was added. There was a marked stimulation of Ca^{2+} uptake by ATP in the presence of oligomycin or atractylate, substances that are known to prevent the delivery of energy from ATP to mitochondrial metabolism.

METHODS

Mitochondria were prepared from rat brain by a modification of the method of OZAWA *et al.*[3]. Brains from four rats were homogenized in a medium composed of 0.3 M mannitol, 0.1 mM EDTA and 0.1 % bovine serum albumin. The homogenate (1 part tissue, 10 parts medium) was centrifuged at 600 $\times$ *g* for 10 min and the supernatant fluid carefully decanted. This fraction was then centrifuged at 10000 $\times$ *g* for 10 min. The sediment was resuspended in 12 ml of homogenization medium and

again centrifuged at 7000 × *g* for 10 min. The sediment (F_1 pellet) from this centrifugation was washed carefully 3 times with about 0.3 ml of medium. The decanted supernatant fluid and the washing solution from this mitochondrial pellet (F_1) were resuspended in the same medium and centrifuged at 7000 × *g* for 10 min in order to obtain a second pellet of mitochondria (F_2). This was done to increase the yield of mitochondria. The second pellet of mitochondria was also washed 3 times with about 0.3 ml of the homogenization medium. This washing solution was discarded. Both pellets (F_1 and F_2) were then resuspended in 12 ml of the same medium and were centrifuged again for 10 min at 7000 × *g*. After the final centrifugation the sediment was washed 3 times with 0.3 ml medium containing 0.3 M mannitol, 0.1 mM histidine and 0.1 % bovine serum albumin. The mitochondria were finally suspended in about 1.8 ml of this medium by gentle homogenization. In order to avoid the presence of a Ca^{2+}-chelating agent in the Ca^{2+}-uptake experiments, histidine was substituted for EDTA in the final suspension medium.

Mitochondria, suspended in the appropriate reaction mixtures, were incubated in beakers at 25° and agitated with magnetic stirrers. Radioactive $CaCl_2$ was added to 12 ml reaction mixture after 1-min preincubation. The amount of $^{45}CaCl_2$ added gave a concentration of 0.1 mM and a radioactivity of about 400 counts/min per ml reaction mixture. Samples were taken at timed intervals and filtered through 0.45 μ Millipore filters. The Ca^{2+} contents of the filtrates were determined by atomic absorption spectrophotometry. In experiments in which $^{45}Ca^{2+}$ was used, radioactivity was determined by a low-background planchet counter (Picker, Model 610222). During the preparation of the mitochondria all solutions were kept at a temperature a few degrees above zero. Oligomycin was obtained from Sigma Chemical Co. and the stock solution was dissolved in absolute alcohol. Atractylate was a gift from Dr. R. Santi.

EXPERIMENTAL RESULTS

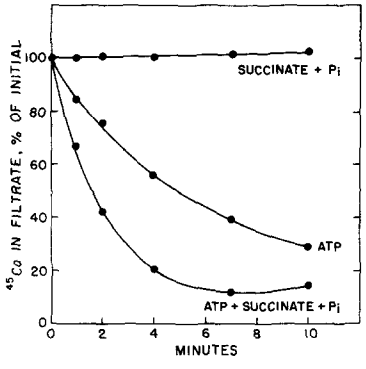
Uptake of Ca^{2+} by brain mitochondria in the presence of succinate and ATP

The results of experiments on the effect of succinate and ATP on the uptake of Ca^{2+} by rat brain mitochondria are recorded in Fig. 1. In the presence of succinate and P_i alone there was no uptake of Ca^{2+}. The addition of ATP alone caused a marked accumulation of Ca^{2+} by the mitochondria. The most rapid uptake of Ca^{2+}, however, was observed to occur in the presence of ATP, P_i and succinate. It appears from these results that, in contrast to observations with mitochondria from other tissues, brain mitochondria require the presence of ATP for respiration-supported Ca^{2+} uptake to occur.

The effect of inhibitors on Ca^{2+} uptake by rat brain mitochondria

Experiments on the effects of the inhibitors of oxidative phosphorylation, oligomycin and atractylate, on Ca^{2+} uptake are presented in Fig. 2. As in the experiment in Fig. 1, there is a rapid uptake of Ca^{2+} by the mitochondria in the presence of ATP. This uptake of Ca^{2+} was abolished by oligomycin, atractylate or both inhibitors added together. When ATP, succinate and P_i were present, the uptake of Ca^{2+} was greater than with ATP alone. Oligomycin or atractylate inhibited Ca^{2+} uptake under these conditions but the ability of the mitochondria to take up Ca^{2+}

was still considerable despite the presence of these inhibitors in concentrations which completely suppressed Ca^{2+} uptake supported by energy from ATP. It is clear from these observations that ATP can facilitate Ca^{2+} transport in brain mitochondria in the presence of an oxidizable substrate by a mechanism that does not involve energy transfer from the nucleotide.



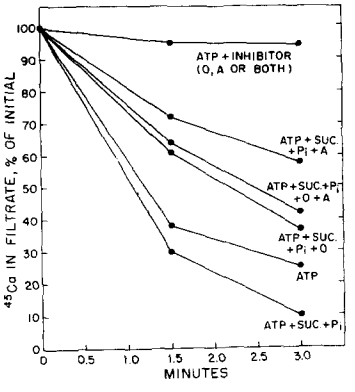


Fig. 1. Ca^{2+} uptake by rat brain mitochondria. Rat brain mitochondria were incubated at 25° in medium of the following composition: 37 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 9.2 mM $MgCl_2$, 3.8 mM ATP, 5.0 mM mannitol, 1.6 μM histidine and 17 μg albumin per ml. When present: 5 mM sodium succinate and 1.7 mM potassium phosphate, KCl to 250 mosM, $^{45}CaCl_2$ added after 1 min at a concentration of 0.1 mM. Mitochondrial protein = 0.097 mg/ml.

Fig. 2. Effect of inhibitors on Ca^{2+} uptake by rat brain mitochondrria. Rat brain mitochondria were incubated at 25° in medium of the following composition: 35 mM HEPES (pH 7.4), 8.8 mM $MgCl_2$, 4.8 mM ATP, 5.0 mM mannitol, 1.6 μM histidine and 17 μg albumin per ml. When present: 6 mM sodium succinate and 2.0 mM potassium phosphate, KCl to 250 mosM. $^{45}CaCl_2$ added after 1 min at a concentration of 0.1 mM. Mitochondrial protein = 0.12 mg/ml. Inhibitors when added: oligomycin (O) 0.33 μg/ml, atractylate (A) 40 μg/ml.

It is interesting that atractylate inhibited Ca^{2+} uptake to a greater extent than oligomycin. However, when both inhibitors were present, the degree of inhibition was that produced by oligomycin alone. In effect, oligomycin prevented atractylate from exerting its maximum inhibition of Ca^{2+} transport. We have no explanation for this interesting phenomenon.

The results of three additional experiments were in complete accord with the observations reported in Fig. 2. In one experiment Ca^{2+} disappearance from the medium was determined both by absorption spectrophotometry and by the determination of radioactivity and identical results were obtained. In another experiment, two concentrations of atractylate were used. The higher concentration was two times that used in the experiment in Fig. 2, but this amount of inhibitor did not lead to further inhibition of Ca^{2+} transport.

DISCUSSION

The preparations of brain mitochondria used here had no measurable ATPase activity under the conditions employed in the Ca^{2+}-uptake experiments. Their respiratory control values were about 3 with succinate as substrate. This is comparable

to the value of 3.8 obtained by OZAWA *et al.*[3] with the same substrate but in the absence of added Mg^{2+}.

Results of the experiments on ion uptake demonstrate that brain mitochondria have the ability to accumulate Ca^{2+} and that this process can be supported by energy from ATP or from respiration. ATP has a dual role; it can by itself support Ca^{2+} entrance into brain mitochondria but it also allows energy from respiration to be utilized for mitochondrial Ca^{2+} uptake. There are several possible explanations for this latter action of ATP. It is possible that the nucleotide forms a complex with Ca^{2+} on the mitochondrial membranes that is necessary for respiration-dependent Ca^{2+} uptake. However, atractylate has been found to inhibit binding of ATP by mitochondria[4,5] but the inhibitor did not prevent ATP from facilitating Ca^{2+} uptake in the presence of succinate. Another possibility is that ATP exerts an effect on respiration-dependent Ca^{2+} transport by chelating Ca^{2+} and preventing damaging effects of free Ca^{2+} on mitochondrial membranes. Further experiments are needed to establish the mechanism by which ATP can increase Ca^{2+} uptake by mitochondria by a mechanism not involving the transfer of energy.

The importance *in vivo* of Ca^{2+} transport by brain mitochondria is at present a matter for speculation. BLAUSTEIN AND HODGKIN[6] recently showed that in squid axon CN^- produced a 30-fold increase in the concentration of diffusable Ca^{2+} and postulated that Ca^{2+} is normally held in vesicles or mitochondria and that ATP is required to maintain the Ca^{2+} in these organelles. Our data demonstrate that mitochondria from nervous tissue have a marked capacity to take up Ca^{2+} and that ATP plays a vital role in this process.

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