Biochimica et Biophysica Acta, 307 (1973) 599–606

BBA 76323

THE EFFECT OF PHOSPHOENOLPYRUVATE ON CALCIUM TRANSPORT BY MITOCHONDRIA

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(Received December 8th, 1972)

SUMMARY

Phosphoenolpyruvate was found to inhibit net uptake of Ca^{2+} by rat heart and liver mitochondria. The main action of phosphoenolpyruvate is to increase the rate of efflux of mitochondrial Ca^{2+}. The effect of phosphoenolpyruvate on mitochondrial Ca^{2+} transport is antagonized by ATP and by atractylate and is observed when mitochondria are respiring in the presence of NAD-linked substrates such as glutamate and pyruvate *plus* malate. In liver mitochondria phosphoenolpyruvate is also effective in the presence of succinate but not when rotenone is added. Glycolytic intermediates other than phosphoenolpyruvate had little effect on mitochondrial Ca^{2+} transport.

INTRODUCTION

In a study of the action of various glycolytic intermediates on mitochondrial Ca^{2+} transport we observed that phosphoenolpyruvate inhibited Ca^{2+} uptake by heart mitochondria incubated *in vitro* but that other glycolytic intermediates studied had little or no effect on this process[1]. McCoy and Doeg[2] have reported that phosphoenolpyruvate also inhibited *in vitro* protein synthesis by liver mitochondria. In the present publication we present results of experiments on the action of phosphoenolpyruvate on Ca^{2+} transport by liver and heart mitochondria which show that the main action of phosphoenolpyruvate is to produce Ca^{2+} efflux in the presence of respiratory substrates linked to the reduction of NAD.

METHODS

Preparation of mitochondria

Mitochondria were prepared from rat hearts by the method of Von Korff[3]. The final suspension of mitochondria was in 0.18 M KCl. Rat liver mitochondria in 0.250 M sucrose were prepared by the method of Hogeboom[4] as described by Myers and

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TMPD, tetramethylphenylenediamine.

Slater[5]. The protein concentrations were determined with biuret according to Cleland and Slater[6].

Incubation

Mitochondria suspended in the appropriate reaction media were incubated at 26 °C in small beakers stirred with rotating magnets and open to the air. The total volumes of the mixtures were 4–5 ml and 1-ml samples were removed at timed intervals and filtered through Millipore filters with pore diameters of 0.45 or 0.65 μm.

Preparation of Ca^{2+}-loaded mitochondria

In experiments in which Ca^{2+} extrusion was followed, heart mitochondria were preloaded with Ca^{2+} as follows: freshly prepared mitochondria (3–4 ml) were added to 10 ml solution containing 0.040 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4), 0.010 M $MgCl_2$ and 0.080 M KCl. 2 ml 0.1 M sodium glutamate, 1.5 ml 0.02 M potassium phosphate and 1 ml 0.02 M $CaCl_2$ were then added and the mixture incubated with stirring at 26 °C for 5 min. The mitochondria were removed by centrifugation at 12000 × *g* for 5 min and washed twice with 0.18 M KCl by resuspension and centrifugation. They were finally suspended in 0.18 M KCl by gentle homogenization. Although the Ca^{2+} concentration (1.1 mM) in the loading experiments was high, the mitochondrial content was twice as high as in the other experiments so that the amount of Ca^{2+} per mg of mitochondria was about the same in all experiments performed.

Determination of Ca^{2+}

The solutions obtained after Millipore filtration were diluted about 1/12 with 0.1 M KCl–0.01 M $SrCl_2$ and the Ca^{2+} concentrations determined by atomic absorption spectroscopy (Perkin-Elmer Model 290B).

Chemicals

HEPES, dithiothreitol and phosphoenolpyruvate (as the potassium or tricyclohexylammonium salt) were obtained from Calbiochem and oligomycin from Sigma Chemical Co. Atractylate was a gift from Dr R. Santi.

RESULTS

Effect of phosphoenolpyruvate on respiration-supported calcium uptake by rat heart mitochondria

Experimental results illustrating the effect of phosphoenolpyruvate on Ca^{2+} uptake by heart mitochondria in the presence of glutamate and inorganic phosphate are recorded in Fig. 1. In these experiments phosphoenolpyruvate was added 1 min before $CaCl_2$.

A significant inhibition of Ca^{2+} uptake was observed when as little as 0.12 mM phosphoenolpyruvate was present and the degree of inhibition increased as the concentration of phosphoenolpyruvate was raised. It is important to note that the initial rate of Ca^{2+} uptake was little affected and that the inhibition of net uptake of Ca^{2+} increased as the incubation proceeded. This was substantiated in a similar experiment in which the Ca^{2+} concentration in the medium was determined at 30-s intervals. In

other experiments it was observed that the inhibitory effects of small concentrations of phosphoenolpyruvate were enhanced when the mitochondria were pre-exposed to phosphoenolpyruvate for more than 1 min. Similar results were obtained with the potassium and tricyclohexylammonium salts of phosphoenolpyruvate.

Other glycolytic intermediates were also tested. Fructose 1,6-diphosphate, glucose 6-phosphate and glucose 1-phosphate were found to be ineffective at concentrations as high as 2.4 mM. A small but significant inhibition was observed with 1.0 mM 3-phosphoglyceric acid, possible caused by conversion of this substance to phosphoenolpyruvate by glycolytic enzymes present in the mitochondrial preparations.

Reversal of the effect of phosphoenolpyruvate by ATP and atractylate

Experimental results showing the antagonistic actions of ATP and atractylate on the effect of phosphoenolpyruvate on Ca^{2+} uptake by heart mitochondria are recorded in Fig. 2.

In these experiments the mitochondria were preincubated for 1 min before addition of $CaCl_2$. ATP, phosphoenolpyruvate or atractylate were added initially as indicated. It is seen that 0.47 mM ATP completely overcame the inhibitory effect of

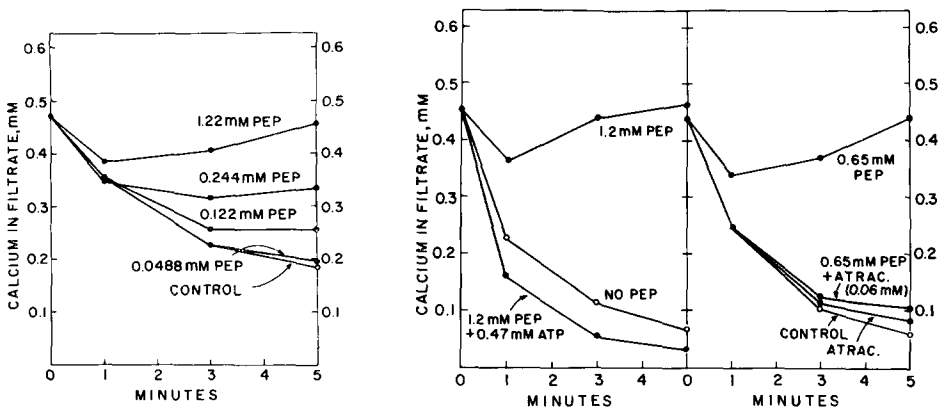

Fig.: 1 Effect of phosphoenolpyruvate on Ca^{2+} uptake by rat heart mitochondria. Composition of reaction system: 29 mM HEPES (pH 7.4), 7.3 mM $MgCl_2$, 7.3 mM sodium glutamate, KCl to 250 mosM, 0.5 mM potassium phosphate (pH 7.4) and phosphoenolpyruvate as indicated. 0.47 mM $CaCl_2$ added after 1 min of preincubation with and without phosphoenolpyruvate. 0.67 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 4.1 ml. PEP, phosphoenolpyruvate.

Fig. 2. Left panel. Reversal of action of phosphoenolpyruvate by ATP with rat heart mitochondria. Composition of reaction systems: 29 mM HEPES (pH 7.4), 7.2 mM $MgCl_2$, 7.2 mM sodium glutamate, KCl to 250 mosM, 0.72 mM potassium phosphate (pH 7.4), phosphoenolpyruvate and ATP as indicated. 0.45 mM $CaCl_2$ added after 1 min of preincubation with and without phosphoenolpyruvate. ATP, when present, added at the same time as phosphoenolpyruvate. 0.94 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 4.12 ml. Right panel. Reversal of the action of phosphoenolpyruvate by atractylate with rat heart mitochondria. Composition of reaction system 29 mM HEPES (pH 7.4), 7.2 mM $MgCl_2$, 7.2 mM sodium glutamate, KCl to 250 mosM. 0.72 mM potassium phosphate (pH 7.4), phosphoenolpyruvate and atractylate as indicated, 0.44 mM $CaCl_2$ added 1 min after preincubation with or without inhibitors. When both phosphoenolpyruvate and atractylate were present they were added at the same time. 0.51 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 4.19 ml. PEP, phosphoenolpyruvate; ATRAC., atractylate.

1.2 mM phosphoenolpyruvate (left panel of Fig. 2). In other experiments, it was shown that oligomycin or atractylate did not interfere with the action of ATP in preventing the effect of phosphoenolpyruvate on calcium uptake. In similar experiments it was observed that ITP or IMP at concentrations of 0.24 mM were totally ineffective in opposing the action of phosphoenolpyruvate.

Results from experiments on the action of atractylate on heart mitochondria incubated with glutamate and phosphate but no ATP are recorded in the right panel of Fig. 2. It is seen that 0.06 mM atractylate completely overcame the effect of 0.65 mM phosphoenolpyruvate. Actually smaller concentrations of atractylate were equally effective since 6 μM of the inhibitor also abolished the effect of 0.65 mM phosphoenolpyruvate.

ATP itself is very potent in antagonizing the action of phosphoenolpyruvate as seen from the experiments reported in the left panel of Fig. 3.

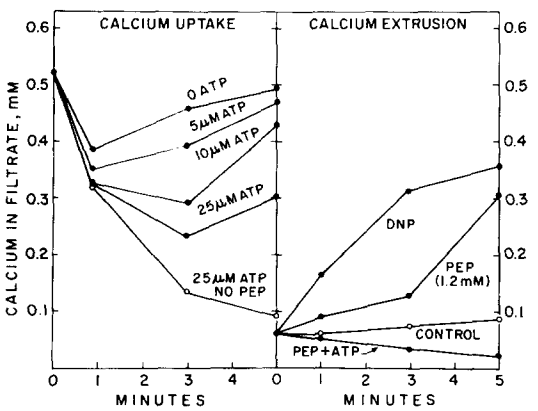

Fig. 3. Left panel. Inhibition of the action phosphoenolpyruvate by different concentrations of ATP with rat heart mitochondria. Composition of reaction system: 29 mM HEPES (pH 7.4), 7.3 mM $MgCl_2$, 7.3 mM sodium glutamate, KCl to 250 mosM, 0.73 mM potassium phosphate (pH 7.4) 1.22 mM phosphoenolpyruvate and ATP as indicated. $CaCl_2$ (0.52 mM) added after 1 min of preincubation with and without phosphoenolpyruvate. ATP, when present, added at the same time as phosphoenolpyruvate. 0.79 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 4.1 ml. Right panel. Extrusion of Ca^{2+} from Ca^{2+}-loaded rat heart mitochondria. Composition of reaction system: 31 mM HEPES (pH 7.4), 7.7 mM $MgCl_2$, 7.7 mM sodium glutamate, KCl to 250 mosM. When present, 1.2 mM phosphoenolpyruvate, 0.51 mM ATP or 41 μM dinitrophenol. Phosphoenolpyruvate (with and without ATP) and dinitrophenol were added after 1 min preincubation. Mitochondria were loaded with Ca^{2+} as described in Methods. 1.07 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 3.9 ml. PEP, phosphoenolpyruvate; DNP, dinitrophenol.

When the incubations were carried out in the presence of 1.22 mM phosphoenolpyruvate as little as 5 μM ATP had a significant effect in alleviating the inhibition caused by phosphoenolpyruvate and increasing concentrations of ATP further overcame the action of phosphoenolpyruvate

Effects of phosphoenolpyruvate on Ca^{2+}-loaded mitochondria

The observation that phosphoenolpyruvate had little or no effect on the initial rate of uptake of Ca^{2+} but exerted its effect only after some mitochondrial Ca^{2+}

uptake had taken place suggested that the action of phosphoenolpyruvate was that of increasing the rate of efflux of Ca^{2+}. To test this possibility, heart mitochondria pre-loaded with Ca^{2+} as described in Methods were incubated in a medium containing glutamate. In controls the Ca^{2+} content of the mitochondria was well maintained but addition of phosphoenolpyruvate caused extrusion of the accumulated Ca^{2+} (right panel of Fig. 3). The effect of phosphoenolpyruvate on Ca^{2+} efflux although marked was slow in onset compared to the action of 2,4-dinitrophenol on this process. The addition of ATP completely reversed the effect of phosphoenolpyruvate.

Effect of substrate on the action of phosphoenolpyruvate

The experiments so far reported were carried out with heart mitochondria incubated with glutamate as the respiratory substrate. The results of experiments with different substrates are reported in Table I.

TABLE I

ROLE OF SUBSTRATE IN THE INHIBITORY EFFECT OF PHOSPHOENOLPYRUVATE ON Ca^{2+} UPTAKE BY RAT HEART MITOCHONDRIA

Composition of reaction system: 30 mM HEPES (pH 7.4), 7.4 mM $MgCl_2$, 0.50 mM potassium phosphate, KCl to 250 mosM. When present, 1.22 mM phosphoenolpyruvate, 7.4 mM sodium glutamate, 3.7 mM sodium malate, 3.7 mM sodium pyruvate, 7.4 mM sodium succinate, 3.7 mM ascorbate and 0.250 mM TMPD. $CaCl_2$ (0.51 mM) added after 1 min of preincubation with and without phosphoenolpyruvate. 1.28 mg mitochondrial protein per ml. Temperature 26 °C. Total volume 4.05 ml.

Substrate	*% inhibition at 3 min*	*% inhibition at 5 min*
Glutamate	77	94
Succinate	0	2
Malate + pyruvate	28	64
Ascorbate + TMPD	6	9.5

It is seen that the degree of inhibition of net uptake of Ca^{2+} was large with glutamate as substrate, somewhat smaller with malate *plus* pyruvate and almost absent with succinate or ascorbate *plus* tetramethylphenylenediamine (TMPD). These findings indicate that the action of phosphoenolpyruvate involves reactions concerned with the first energy-conserving site of the respiratory chain.

Effect of phosphoenolpyruvate on Ca^{2+} uptake by liver mitochondria

Results of experiments on the effect of phosphoenolpyruvate on Ca^{2+} uptake by rat liver mitochondria are reported in Table II. As with heart mitochondria, phosphoenolpyruvate markedly inhibited net Ca^{2+} uptake in the presence of glutamate and phosphate. However, in contrast to the findings with heart mitochondria, Ca^{2+} uptake was also strongly inhibited when succinate was the respiratory substrate. We then carried out similar experiments with rotenone present. Under these conditions, when endogenous NADH oxidation was inhibited, phosphoenolpyruvate had

no effect on mitochondrial calcium uptake (Table II) again indicating that the action of phosphoenolpyruvate involves reactions in the early part of the electron transport chain.

TABLE II

THE EFFECT OF PHOSPHOENOLPYRUVATE ON Ca^{2+} UPTAKE BY RAT LIVER MITOCHONDRIA

Composition of reaction system: 30 mM HEPES (pH 7.4), 7.5 mM $MgCl_2$, 7.5 mM sodium glutamate or succinate, 0.75 mM potassium phosphate, KCl to 250 mosM, 0.030 M sucrose. Phosphoenolpyruvate concentration as indicated. $CaCl_2$ added after 1 min preincubation (0.44 mM in Expt 1, 0.47 mM in Expt 2). Rotenone 5.0 μg/ml, when present. Temperature 26 °C. Mitochondrial protein 1.16 mg/ml in Expt 1, 1.48 mg/ml in Expt 2. Total volume 4.15 ml in Expt 1, 4.04 ml in Expt 2.

Expt No.	*Substrate*	*Phosphoenol-pyruvate (mM)*	*Ca^{2+} uptake (μmoles/ml)*	
			1 min	*5 min*
1	Glutamate	0	0.281	0.389
	$+P_i$	0.60	0.220	0.121
2	Succinate	0	0.320	0.446
	$+P_i$	0.67	0.144	0.091
	Succinate	0	0.458	0.458
	$+P_i$ + rotenone	0.67	0.458	0.458

It is interesting that rotenone markedly increased Ca^{2+} uptake by liver mitochondria respiring in the presence of succinate. This is general phenomenon that we have observed consistently in many experiments and may be related to inhibition of malate oxidation by rotenone.

DISCUSSION

The results presented here show that an intermediate of glycolysis can influence an important cellular function of mitochondria, the uptake and release of Ca^{2+}. Phosphoenolpyruvate appears to be unique in this respect, since other glycolytic intermediates had little or no effect on mitochondrial Ca^{2+} transport.

The mechanism of the effect of phosphoenolpyruvate on mitochondrial Ca^{2+} transport is not clear at present. One possibility that we considered was that phosphoenolpyruvate acts as a substrate for the pyruvate kinase reaction, phosphoenolpyruvate + ADP = Pyruvate + ATP, and in this way removes ADP present between the inner and outer mitochondrial membranes. This does not happen since in experiments with heart mitochondria incubated *in vitro* we found that phosphoenolpyruvate was not metabolized during a 3-min incubation even in the presence of excess ADP. Another possibility is that phosphoenolpyruvate inhibits the oxidation of mitochondrial NADH. However, experiments with liver mitochondria exposed to an osmotic shock (five times dilution of the original suspension with water) gave no support to this hypothesis since phosphoenolpyruvate in concentrations up to 5 mM caused a slight increase rather than a decrease in the rate of oxidation of added NADH. With intact liver mitochondria phosphoenolpyruvate does not influence State 3 or State 4

respiration and inhibits 2,4-dinitrophenol-stimulated respiration in the presence of glutamate to only a small extent.

The fact that the effects of phosphoenolpyruvate described here are seen when the mitochondria are respiring in the presence of substrates giving rise to the formation of NADH suggests that the mechanisms of the action of phosphoenolpyruvate involves an interaction with the process of energy conservation at Site I. Since atractylate and ATP are so effective in overcoming the effects of phosphoenolpyruvate, this compound appears to combine with the same site as atractylate and ATP, presumably the adenine nucleotide translocator on the inner mitochondrial membrane. Phosphoenolpyruvate most probably does not exert its action intramitochondrially since the tricarboxylate translocator, which is involved in transport of phosphoenolpyruvate across the inner mitochondrial membrane[8], is absent in heart mitochondria[9]. The effectiveness of atractylate in opposing the action of phosphoenolpyruvate also supports this conclusion.

Phosphoenolpyruvate does not appear to act by interfering with phosphate transport since dithiodinitrobenzoic acid, a potent inhibitor of phosphate uptake by mitochondria[7], does not cause extrusion of Ca^{2+} from mitochondria and increasing the phosphate concentration of the medium did not diminish the action of phosphoenolpyruvate. Vignais *et al.*[10] have proposed that atractylate, its aglycone, atractylegenin, and other substances with similar action produce their effects by combining with an allosteric effector site that is part of the adenine nucleotide translocator. One can speculate that phosphoenolpyruvate also acts in a similar manner. It should be noted in this connection that phosphoenolpyruvate contains a methylene group, a structure in the atractylate molecule necessary for full activity of this compound[11].

The observation of McCoy and Doeg[2] that mitochondrial protein synthesis is inhibited by phosphoenolpyruvate indicates that phosphoenolpyruvate may influence several mitochondrial reactions. The inhibition of protein synthesis reported by these authors was found at somewhat higher concentrations of phosphoenolpyruvate than the effects observed here but in both cases the action of phosphoenolpyruvate was overcome by atractylate or ATP indicating that the same site of action may be involved.

Whatever the mechanism of action of phosphoenolpyruvate the most important aspect of the problem is the possibility that changes in glycolytic flux can influence mitochondrial reactions in the intact cell. For phosphoenolpyruvate to be effective in doing so it must reach the mitochondrion at a time which the ATP concentration in the immediate vicinity is low. Further experimentation is needed to determine whether changes in the concentration of phosphoenolpyruvate could play a physiological role in the regulation of mitochondrial metabolism and function.

ACKNOWLEDGMENTS

These studies were supported by a grant from the National Institutes of Health, U. S. Public Health Service (HL-01813). P. C. was supported by a predoctoral fellowship from the Rockefeller Foundation. The experimental data presented here are taken from a thesis by P. C. in partial fulfillment of the degree of Ph. D. in Pharmacology at the University of Pennsylvania. We thank Professor R. Santi for his generous gift of a sample of atractylate used in this work.

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