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FURTHER STUDIES ON THE EFFECT OF PHOSPHOENOLPYRUVATE ON RESPIRATION-DEPENDENT CALCIUM TRANSPORT BY RAT HEART MITOCHONDRIA

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SUMMARY

Phosphoenolpyruvate was found to depress extra oxygen consumption associated with Ca^{2+}-induced respiratory jump by rat heart mitochondria. Addition of phosphoenolpyruvate to mitochondria which have accumulated Ca^{2+} in the presence of glutamate and inorganic phosphate causes the release of Ca^{2+} from mitochondria. The phosphoenolpyruvate-stimulated Ca^{2+} efflux can be observed with mitochondria loaded with low initial Ca^{2+} concentration (0.12 mM) in the incubation medium. Measurements of mitochondrial H^+ translocation produced by addition of Ca^{2+} to respiring mitochondria show that phosphoenolpyruvate depresses H^+ ejection and enhances H^+ uptake by mitochondria. The Ca^{2+}-releasing effect of phosphoenolpyruvate was found to be significantly stronger than that produced by rotenone when added to mitochondria loaded with Ca^{2+} in the presence of glutamate and inorganic phosphate. Dithiothreitol cannot overcome the effect of phosphoenolpyruvate on mitochondrial Ca^{2+} transport.

INTRODUCTION

In previous publications we have reported that phosphoenolpyruvate inhibits net uptake of Ca^{2+} by rat heart mitochondria incubated in vitro but that other glycolytic intermediates studied have little or no effect on this process [1, 2]. The effect of phosphoenolpyruvate on mitochondrial Ca^{2+} transport is antagonized by ATP and by atractyloside and is observed when mitochondria are respiring in the presence of NAD-linked substrates [2]. Phosphoenolpyruvate has also been found to inhibit in vitro protein synthesis by liver mitochondria and this inhibition is overcome by adding ADP or atractyloside [3]. The main action of phosphoenolpyruvate on mitochondrial Ca^{2+} transport appears to be the stimulation of Ca^{2+} efflux since addition of phosphoenolpyruvate to Ca^{2+}-loaded rat heart mitochondria greatly enhances the release of Ca^{2+} from mitochondria [2]. However, in this experi-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

ment the mitochondria were pre-loaded in the presence of relatively high calcium concentration (1.1 mM) which may produce structural as well as functional damage to mitochondrial membranes [4, 5]. In the present paper the effects of phosphoenolpyruvate on Ca^{2+} transport and Ca^{2+}-induced respiratory jump and H^+ translocation by rat heart mitochondria were investigated. The results show that phosphoenolpyruvate stimulates Ca^{2+} efflux by rat heart mitochondria and this action can be observed with mitochondria loaded with small initial Ca^{2+} concentration in the incubation medium.

METHODS AND MATERIALS

Rat heart mitochondria were prepared by the method of Von Korff [6]. The final suspension of mitochondria was in 0.18 M KCl. The protein concentrations were determined with biuret according to Cleland and Slater [7]. In the experiments involving measurements of Ca^{2+} concentrations or H^+ translocation, the mitochondria suspended in appropriate reaction media were incubated in small beakers stirred with rotating magnets and open to the air. Changes in H^+ concentrations were determined with a sensitive glass electrode connected to a pH meter. Ca^{2+} concentrations were determined, after Millipore filtration of the reaction mixtures at timed intervals, by atomic absorption spectroscopy (Perkin-Elmer Model 290B). In the cases where $^{45}CaCl_2$ was employed the radioactivity was determined by a low-background planchet counter (Picker, Model 610222). Measurements of oxygen consumption were carried out in the chamber of a Gilson oxygraph equipped with a Clark oxygen electrode. The rates of oxygen consumption were expressed as μatom O/ml per min. Unless indicated otherwise the temperature was 26 °C in all experiments. Experimental conditions are reported in the legends to the figures.

N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), dithiothreitol and phosphoenolpyruvate were obtained from Calbiochem and rotenone from Sigma Chemical Co.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of phosphoenolpyruvate on Ca^{2+}-stimulated respiration by rat heart mitochondria respiring in the presence of glutamate and inorganic phosphate. It is seen that preincubation of mitochondria with phosphoenolpyruvate caused some inhibition on the initial rate of Ca^{2+}-induced respiratory jump and the jump declined earlier than did the control response. Measurements of the extra oxygen uptake at 2 min after adding $CaCl_2$ showed that phosphoenolpyruvate depressed the amount of extra oxygen consumption to about half of the control value. In fact the reduction of extra oxygen uptake by phosphoenolpyruvate can be observed at any timed interval following the addition of $CaCl_2$. This observation indicated the inhibition by phosphoenolpyruvate of respiration-dependent calcium uptake by mitochondria [8, 9]. It is interesting to point out that although the concentration of phosphoenolpyruvate used in this experiment has been reported to cause almost complete inhibition of net Ca^{2+} uptake by mitochondria [2], the initial rates of Ca^{2+} uptake [2] and Ca^{2+}-stimulated respiration were relatively unaffected by such high concentration of this compound.

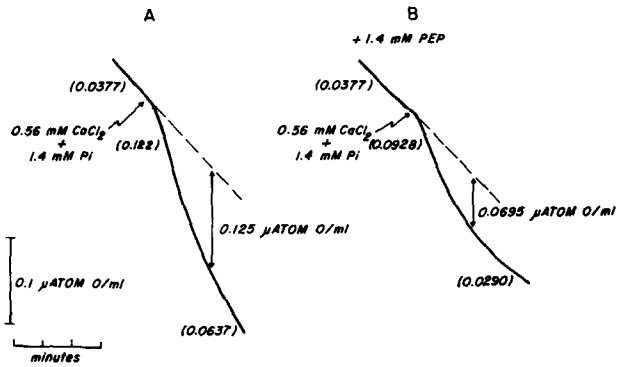
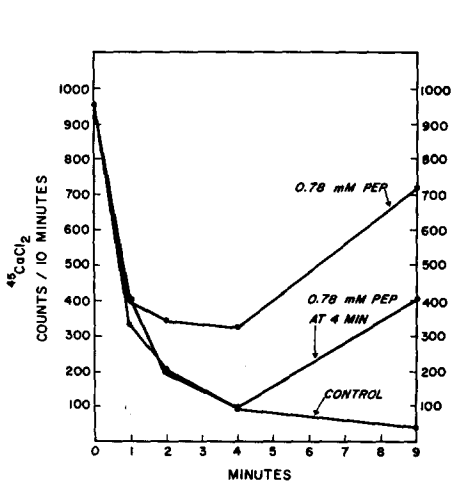


Fig. 1. Inhibition by phosphoenolpyruvate of the Ca^{2+}-stimulated respiration by rat heart mitochondria. Composition of reaction system: 34 mM HEPES buffer, pH 7.4, 8.4 mM $MgCl_2$, 8.4 mM potassium glutamate and KCl to 250 mosM. Phosphoenolpyruvate (PEP), $CaCl_2$ and inorganic phosphate as indicated. The figures in parentheses represent the rates of oxygen uptake in μatom O/ml per min. The figures at the vertical arrows denote the amounts of extra oxygen consumed at 2 min after the addition of $CaCl_2 + P_i$ · 1.37 mg mitochondrial protein per ml. Total volume 1.43 ml.

In order to demonstrate the ability of phosphoenolpyruvate to induce Ca^{2+} efflux from mitochondria loaded with low initial Ca^{2+} concentration, experiments using $^{45}CaCl_2$ were performed and the results are recorded in Fig. 2. In these experiments $^{45}CaCl_2$ was added to give the initial concentration of 0.12 mM. In the absence of phosphoenolpyruvate rat heart mitochondria removed Ca^{2+} from the medium so that about one-twentieth of total radioactivity, corresponding to 6 μM Ca^{2+}, remained in the medium after 9 min of incubation. Preincubation of mitochondria with phosphoenolpyruvate for 1 min before the addition of $^{45}CaCl_2$ had practically no effect on the first minute Ca^{2+} uptake but later on the uptake of Ca^{2+} was gradually inhibited and the accumulated Ca^{2+} was finally extruded. Similar Ca^{2+}-releasing action of phosphoenolpyruvate was also observed when this compound was added to mitochondria which had accumulated Ca^{2+} for 4 min. It is clear from these experiments that phosphoenolpyruvate not only inhibits Ca^{2+} uptake but also stimulates the release of Ca^{2+} from rat heart mitochondria, and these effects can be observed with mitochondria incubated in medium containing Ca^{2+} at concentrations which do not produce deleterious actions on mitochondrial structures and functions [5].

Several investigators have shown that liver or heart mitochondria suspending in medium supplemented with respiratory substrates or ATP in the presence or absence of permeant anions respond to the addition of $CaCl_2$ with the ejection of H^+ as Ca^{2+} is removed from the medium and, conversely, extrusion of Ca^{2+} from mitochondria is associated with the disappearance of H^+ from the medium [5, 10]. The changes in H^+ concentrations in the medium can be detected with a sensitive glass electrode. Since phosphoenolpyruvate was found to inhibit the uptake and stimulate the release of Ca^{2+} by rat heart mitochondria, the corresponding changes in H^+ movements were anticipated. Results of experiments on the action of phosphoenolpyruvate on Ca^{2+}-induced H^+ translocation by rat heart mitochondria



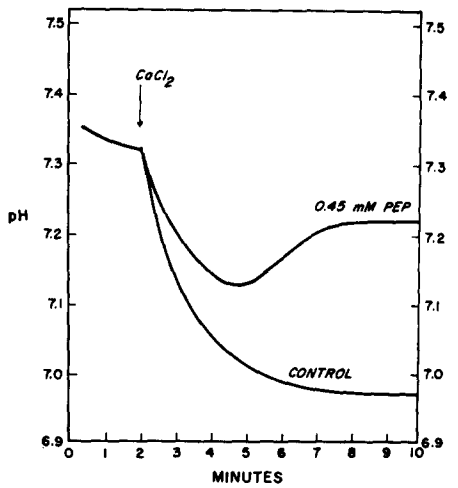


Fig. 2. The effect of phosphoenolpyruvate on Ca^{2+} transport by rat heart mitochondria. Composition of reaction system: 31.4 mM HEPES buffer, pH 7.4, 8 mM $MgCl_2$, 6 mM potassium glutamate, 0.80 mM potassium phosphate and KCl to 250 mosM. $^{45}CaCl_2$ was added to give the initial concentration of 0.12 mM. Phosphoenolpyruvate (PEP), when present, was added 1 min before or 4 min after the addition of $^{45}CaCl_2$. 0.85 mg mitochondrial protein per ml. Total volume 5.1 ml.

Fig. 3. The effect of phosphoenolpyruvate (PEP) on Ca^{2+}-induced H^+ translocation by rat heart mitochondria. Composition of reaction system: 8.85 mM potassium glutamate, 8.85 mM $MgCl_2$, 0.85 mM potassium phosphate and KCl to 250 mosM. $CaCl_2$ (0.17 mM) was added after 2 min of preincubation with and without phosphoenolpyruvate (0.45 mM). 0.69 mg mitochondrial protein per ml. Total volume 5.65 ml. The experiments were carried out at room temperature.

respiring in the presence of glutamate and inorganic phosphate are presented in Fig. 3. In these experiments mitochondria were preincubated with and without phosphoenolpyruvate for 2 min before $CaCl_2$ (0.17 mM) was added. In control experiments addition of $CaCl_2$ caused mitochondrial H^+ ejection which was detected as a fall in pH of the medium. When phosphoenolpyruvate was present, the decrease in pH was depressed and the pH began to rise again at the end of the fifth minute. The increase in pH continued to about the end of eighth minute and remained unchanged up to the tenth minute. Obviously the depression of the decrease in pH reflected inhibition of mitochondrial Ca^{2+} uptake whereas the rise in pH indicated stimulation of mitochondrial Ca^{2+} efflux by phosphoenolpyruvate. Note also that phosphoenolpyruvate had only small effect on the initial rate of the decline in pH. Determinations of Ca^{2+} at the end of experiments showed that more than 80 % of the initial Ca^{2+} was taken up in the control while less than 10 % was accumulated in the presence of phosphoenolpyruvate. These observations provide additional evidence that phosphoenolpyruvate inhibits Ca^{2+} accumulation and stimulates Ca^{2+} release by mitochondria. In other experiments not reported here dithiothreitol, the reagent known to protect sulfhydryl groups [11, 12], was found to be inactive against the action of phosphoenolpyruvate on mitochondrial Ca^{2+} transport suggesting that phosphoenolpyruvate did not produce its effect by combining with mitochondrial sulfhydryl groups.

It has been shown earlier that the effect of phosphoenolpyruvate on mitochondrial

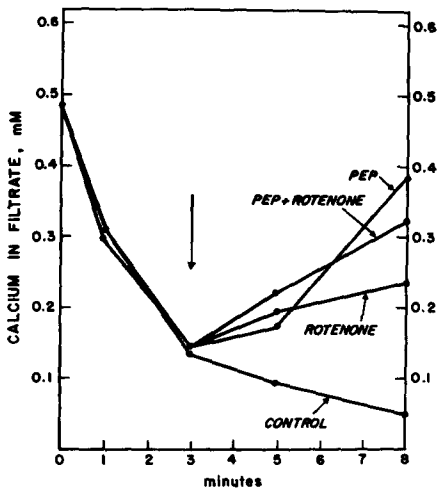


Fig. 4. Comparison of the effects of phosphoenolpyruvate (PEP) and rotenone on Ca^{2+} transport by rat heart mitochondria. Composition of the reaction system: 31 mM HEPES buffer, pH 7.4, 7.7 mM $MgCl_2$, 5.8 mM potassium glutamate, 0.77 mM potassium phosphate and KCl to 250 mosM. When present, 0.62 mM phosphoenolpyruvate and 3.1 μg/ml rotenone. Phosphoenolpyruvate or rotenone was added 3 min after $CaCl_2$. When both phosphoenolpyruvate and rotenone were present, rotenone was added at 3 min and phosphoenolpyruvate 10 s later. 0.81 mg mitochondrial protein per ml. Total volume 5.2 ml.

Ca^{2+} transport can be observed only when mitochondria were respiring in the presence of NAD-linked substrates such as glutamate and malate plus pyruvate [2]. Experiments were performed to compare the Ca^{2+}-releasing effect of phosphoenolpyruvate with that of rotenone, a powerful inhibitor of NADH oxidation. In these experiments the inhibitors were added to rat heart mitochondria which had accumulated Ca^{2+} for 3 min (Fig. 4). Addition of rotenone, at concentration high enough to produce practically complete inhibition of respiratory chain-linked NADH oxidation, caused the release of Ca^{2+} from mitochondria. This observation confirms the previous report by Drahota et al. [13] that respiration-dependent Ca^{2+} accumulation and retention by mitochondria can be blocked by respiratory inhibitors. However, a greater release of Ca^{2+} was observed in the presence of 0.62 mM phosphoenolpyruvate. This finding can be explained on the basis that phosphoenolpyruvate not only inhibits Ca^{2+} uptake but also stimulates Ca^{2+} release whereas rotenone has no direct stimulatory action on Ca^{2+} efflux by mitochondria. The rotenone-induced Ca^{2+} release is secondary to the inhibition of NADH oxidation which blocks the generation of "energy pressure" by respiratory chain. Consequently Ca^{2+} uptake is inhibited and the mitochondria are unable to retain the accumulated Ca^{2+} [5, 13]. Phosphoenolpyruvate was also found to cause stronger stimulation than control of Ca^{2+} release from Ca^{2+}-loaded rat heart mitochondria incubated in medium without repiratory substrate or ATP. How phosphoenolpyruvate enhances the release of Ca^{2+} from mitochondria is unknown at present. Light scattering experiments showed that phosphoenolpyruvate did not cause appreciable swelling of rat heart mitochondria even in the presence of appropriate concentrations of $CaCl_2$ and inorganic phosphate. It is of particular interest that

rotenone significantly reduced the effect of phosphoenolpyruvate on mitochondrial Ca^{2+} efflux. This phenomenon had been consistently observed in many experiments. In other experiments not shown here, two times increase in rotenone concentration did not enhance this antagonistic action of rotenone.

Whether the effect of phosphoenolpyruvate on mitochondrial Ca^{2+} transport could play a physiological role in the regulation of mitochondrial and cellular functions is at present a matter of speculation. The mitochondria from different tissues have been shown to possess the capability to accumulate, with high affinity, a large amount of Ca^{2+} [14]. However, massive loading of mitochondria has damaging effects on oxidative phosphorylation as well as other mitochondrial structures and functions [4, 5]. Therefore, it is logical to assume that under in vivo condition some mechanisms must operate to cause the release of Ca^{2+} from mitochondria which have excessively accumulated Ca^{2+}. At present very little is known about the physiological mechanism of Ca^{2+} release from loaded mitochondria. The ability of phosphoenolpyruvate to induce mitochondrial Ca^{2+} efflux raises the possibility that this compound may play a physiological role in the regulation of mitochondrial Ca^{2+} content. One may further speculate that the Ca^{2+}-induced inhibition of mitochondrial oxidative phosphorylation can lead to a reduction in cytoplasmic ATP level. As a consequence, the rate of glycolysis and the intracellular phosphoenolpyruvate content are elevated [15]. The rise in cytoplasmic phosphoenolpyruvate concentration then stimulates Ca^{2+} efflux with the restoration of normal mitochondrial ATP production. Much further work is needed before the role of phosphoenolpyruvate as one of the physiologic regulators of mitochondrial and cellular functions can be established.

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