REVISTA ESPAÑOLA DE FISIOLOGIA, 36, 403-408, 1980

Effect of Phosphoenolpyruvate on Liver Mitochondrial Calcium Retention from Fed and Fasted Rats

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(Received on January 22, 1980)

M. LUCAS, C. OSUNA and R. GOBERNA. *Effect of Phosphoenolpyruvate on Liver Mitochondrial Calcium Retention from Fed and Fasted Rats.* Rev. esp. Fisiol., 36, 403-408. 1980.

Rat liver mitochondria from fed and 4-day-fasted rats are able to accumulate calcium supported by ATP (energy-driven calcium uptake) or by succinate (respiration-driven calcium uptake). Phosphoenolpyruvate decreases the amount of calcium accumulated in both ways, whether the rats were fasted or fed *ad libitum*. Phosphoenolpyruvate induces a net calcium efflux from mitochondria. The effect of phosphoenolpyruvate is partially reversed by ADP. Phosphoenolpyruvate is also able to inhibit the ATP-driven calcium accumulation in the absence of exogenously added respiratory substrates.

The most probable physiological role played by mitochondrial calcium transport is in the regulation of the concentration of free Ca^{2+} in the cytoplasm and hence in the control of several cellular events. Mitochondrial calcium uptake can be driven by respiratory substrates or by ATP; however, relatively little is known regarding the biochemical factors controlling retention and release of calcium. Several substances have been found to promote release of calcium from respiring mitochondria *in vitro*, including phosphoenolpyruvate (1). It has been suggested that the transport of phosphoenolpyruvate by the adenine-nucleotide translocase system in heart (1) and in liver (1, 8) mitochondria may be directly involved in the mechanism of phosphoenolpyruvate induced calcium ion efflux. The adenine nucleotide translocase is inhibited by long-chain acyl-CoA and this may be the explanation for the differences in the phosphate potential be-

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Abbreviations:

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
PEP: phosphoenolpyruvate.

tween cytoplasm and mitochondria in liver tissue during fasting which lead to a decrease in the ATP/ADP ratio in the cytoplasm while within the mitochondria this ratio is elevated.

The present paper describes experiments that attempt to further knowledge about the effect of phosphoenolpyruvate on mitochondrial calcium transport. With this in mind we studied the effect of phosphoenolpyruvate on calcium accumulation and efflux in liver mitochondria from fed and fasted rats in the presence of ADP plus succinate (respiration-driven calcium uptake) or ATP (ATP-driven calcium uptake).

Materials and Methods

Female Wistar rats weighing 200-300 g were fed *ad libitum* or fasted for 24 or 96 hours with free access to water.

Rat liver mitochondria. The liver from one rat was used in every experiment. Mitochondria were prepared as previously described for rat pancreas (3) with some modifications. In brief, the liver was cut into small pieces and rinsed in a medium consisting of mannitol 210 mmol/l, sucrose 70 mmol/l, HEPES 10 mmol/l, and bovine serum albumin 5 mg/ml, adjusted to pH 7.1. The tissue was homogenized in a Potter-Elvehjem homogenizer at 1,200 rpm and with two strokes of a loose-fitting Teflon pestle. The homogenate was filtered through a double layer of cheesecloth to remove unbroken tissue. The volume was adjusted to 40 ml with the homogenization medium and supplemented with EGTA 0.2 mmol/l. The homogenate was centrifuged at 2,500 *g* for 15 min at 4° C to remove nuclei and debris. Mitochondria were spun down from the supernatant by centrifugation at 6,000 *g* for 20 min at 4° C. The pellet was resuspended in 40 ml of the homogenization medium without EGTA and centrifuged again at 6,000 *g* for 20 min at 4° C. The final pellet was resuspended in 2 ml of a medium consisting of KCl 130 mmol/l, $MgCl_2$ 2 mmol/l, KPO_4H_2 0.2 mmol/l, HEPES 10 mmol/l, and bovine serum albumin 5 mg/ml, adjusted to pH 7.1. This medium was also the incubation medium, supplemented with calcium and different effectors as stated in the individual experiments.

Calcium transport. Uptake and release of radioactive $^{45}Ca^{2+}$ was measured by incubating mitochondria, at a final concentration of 0.8-1.5 mg/ml, in 1 ml of the incubation medium described above. At specified time intervals a 200 μl aliquot of the incubation medium was removed and centrifuged for 30 s in an Eppendorf 3.200 centrifuge. A 100 μl aliquot of the supernatant was pipetted into 10 ml of Scintisol. Radioactivity was measured by liquid scintillation spectrometry. The specific radioactivity, approximately 10 μCi/μmol, of calcium in the incubation medium was determined by counting an uncentrifuged 100 μl sample.

Analytical methods. The activity of glutamate dehydrogenase was measured at 25° C (6) after sonication of mitochondria in an MSE sonicator at middle amplitude during 10 s. The relative specific activity of glutamate dehydrogenase as mitochondrial enzyme marker was used to determine the yielding of mitochondria during the preparation procedure.

Protein was determined by the biuret method (2) using bovine serum albumin as standard.

All experiments were usually carried out at least four times. Data are shown in figures only for mitochondria obtained from 16-hour-fasted rats. Identical results were obtained with liver mitochondria from fed and 4-day-fasted rats.

Chemicals. Phosphoenolpyruvate monosodium salt, ATP, ADP and HEPES were obtained from Boehringer Mann-

heim. $^{45}Ca^{2+}$ was obtained from the Radiochemical Centre, Amersham.

Results

As judged by glutamate dehydrogenase analysis, the yielding of mitochondria during the isolation procedure is approximately 40 % of the content in liver tissue with a specific activity of 0.277 ± 0.046 U/mg of mitochondrial protein, and a relative specific activity of 3.55 referred to the homogenate.

Mitochondria from rat liver accumulate almost all the available calcium (about 300 nmol/mg prot.). This accumulation occurred in the presence of ATP (ATP-driven calcium accumulation) or of succinate (respiration-driven calcium accumulation). The amount of calcium accumulated is decreased by phosphoenol pyruvate in both cases, in the ATP-driven calcium accumulation and in the succinate-driven calcium accumulation (fig. 1). ADP at a concentration of 2.5 mmol/l partially overcomes the effect of phosphoenolpyruvate on succinate-supported calcium transport.

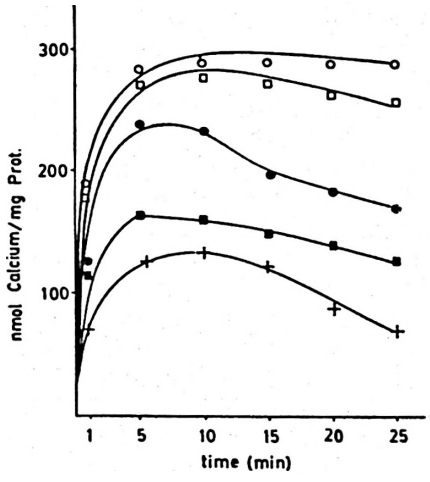

Fig. 1. *Kinetics of mitochondrial calcium accumulation: effect of PEP.*
Calcium accumulation was started by adding $^{45}CaCl_2$ 300 nmol/mg mitochondria after a 5 min preincubation. At indicated time intervals 200 μl aliquots were removed and processed as described under methods. The basic incubation medium was supplemented as follows: 5 mmol/l sucinate (○); 2.5 mmol/l ATP (□); 2 mmol/l PEP in the presence of 5 mmol/l succinate (+); 2 mmol/l PEP in the presence of 2.5 mmol/l ATP (■); 2.5 mmol/l ADP and 2 mmol/l PEP in the presence of 5 mmol/l succinate (●).

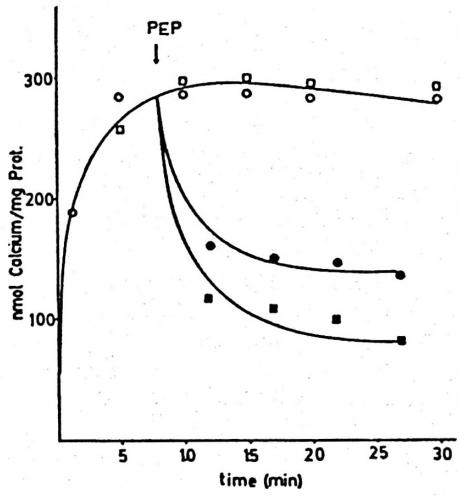

Fig. 2. *Calcium efflux induced by PEP.*
Mitochondria were allowed to accumulate calcium in the presence of 5 mmol/l succinate together with 2.5 mmol/l ADP (○) or in the presence of 2.5 mmol/l ATP (□). PEP was added at minute 10 as indicated by arrow, at a final concentration of 2 mmol/l in test tubes containing 5 mmol/l succinate plus 2.5 mmol/l ADP (●), or ATP 2.5 mmol/l (■). After a 5-min preincubation, the experiment was started by adding $CaCl_2$ 300 nmol/mg prot.

Addition of phosphoenolpyruvate after 10 min, when mitochondrial calcium accumulation reaches a plateau, leads to an immediate decrease of intramitochondrial $^{45}Ca^{2+}$ to less than 50 % of the control value (fig. 2). The kinetics of calcium release shows no difference with respect to the preloading of mitochondria with calcium supported by ATP or succinate plus

ADP, although the extent of calcium release is less in the second case.

Phosphoenolpyruvate was tested in liver mitochondria from fed and 4-day-fasted rats. In both cases the effect of phosphoenolpyruvate shows no difference with respect to the accumulation and efflux of calcium in mitochondria from 16-hour-fasted rats. Phosphoenolpyruvate provokes a decrease in calcium accumulation and a sudden decrease in calcium content from the amount of ion previously loaded, independent of whether the rats were fed or fasted for 4 days (data not shown) or of whether the calcium uptake was driven by ATP or succinate.

Discussion

Although calcium ion uptake by mitochondria is known to be supported both by respiration and ATP hydrolysis, the mechanism of calcium efflux from mitochondria is not well understood.

Succinate alone leads to an accumulation of calcium by mitochondria. In agreement with previous reports (1, 4, 5, 8, 9) the addition of phosphoenolpyruvate decreases the amount of mitochondrial calcium. The addition of ADP to the medium reverses in part the effect of phosphoenolpyruvate on calcium transport. This may be due to the conversion of ADP to ATP which in turn could reverse the effect of phosphoenolpyruvate on respiration-supported calcium transport.

In the absence of exogenously added substrates, i. e., succinate, calcium is accumulated by mitochondria when ATP 2.5 mmol/l is added to the incubation medium. This is also the case for liver mitochondria from 4-day-fasted rats. Under these conditions, phosphoenolpyruvate provokes a decrease in calcium content. It has been described (1, 8) that the effect of phosphoenolpyruvate on mitochondrial calcium transport is antagonized by ATP. In our experiments phosphoenolpyruvate is able to decrease the calcium accumulation supported by ATP and to induce a calcium release from mitochondria to the medium in the presence of ATP 2.5 mmol per liter. The failure of ATP to inhibit the effect of phosphoenolpyruvate may be due to the lack of succinate in the incubation medium and the larger amount of calcium added and accumulated by mitochondria in our experiments. The removal of endogenous ATP by exchange with phosphoenolpyruvate as proposed by PENG *et al.* (4) and supported by ROOS *et al.* (5) may be the essential factor in the induction of calcium release by phosphoenolpyrivuate, but not the only mechanism.

During fasting there is an increase in the mitochondrial [ATP]/[ADP] [P_i] ratio which is decreased after glucose refeeding (7). This has been explained as a consequence of the decreased insulin/glucagon ratio during fasting leading to an accumulation of long-chain acyl-CoA in liver tissue, which might affect the translocation of adenine nucleotides between the cytoplasmic and mitochondrial compartments. Moreover, phosphoenolpyruvate induced calcium efflux is inhibited by palmitoyl-CoA (8). Liver mitochondria from fasting rats show similar patterns of calcium transport. Fasting did not modify the effect of phosphoenolpyruvate on ATP-driven calcium uptake. The accumulation of longchain acyl-CoA during fasting in liver tissue may be overcome during the mitochondrial preparation procedure, and hence it may be expected that it does not affect the phosphoenolpyruvate modification of calcium transport indicating a moment-to-moment control of calcium transport.

Resumen

Las mitocondrias de hígado de ratas alimentadas o sometidas a 4 días de ayuno acumulan calcio en presencia de ATP (captación de calcio dependiente de energía) o de succi-

nato (captación de calcio dependiente de la respiración). El fosfoenolpiruvato disminuye la cantidad de calcio acumulada en ambas formas, independientemente de que las ratas estén en ayuno o alimentadas, e induce una salida neta de calcio de las mitocondrias. El efecto del fosfoenolpiruvato es parcialmente revertido por ADP y también es capaz de inhibir la acumulación de calcio dependiente de ATP, en ausencia de sustratos de la respiraciós.

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