THE EFFECT OF PHOSPHOENOLPYRUVATE ON THE RETENTION OF CALCIUM BY LIVER MITOCHONDRIA

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1. Introduction

The ability of mitochondria to accumulate large amounts of Ca^{2+} via a specific carrier in the inner membrane is extensively documented [1,2]. The release of Ca^{2+} from mitochondria of heart and some other excitable tissues probably occurs via a second carrier, that exchanges Na^+ for Ca^{2+} [3,4]. The mechanism of Ca^{2+} release from other mitochondria that lack the Na^+–Ca^{2+} exchange, e.g., liver is obscure. Some [5–7] have presented evidence for a second Ca^{2+} carrier in liver mitochondria, albeit not exchanging Na^+, which catalyses release, whereas others [8] have concluded that efflux occurs by reversal of the influx process. In addition, considerable attention has been given to the release of Ca^{2+} from mitochondria by exogenously added phosphoenolpyruvate (PEP) [9–11]. However, whether or not the phenomenon of the PEP-induced release of Ca^{2+} has the qualities of a physiological release mechanism, has not been investigated thoroughly. This is the subject of the present paper. In particular, it is shown that the phenomenon is associated with a collapse of the electrical potential difference across the inner membrane, which permits release of Ca^{2+} via reversal of the energy-linked influx process.

2. Materials and methods

2.1. *Preparation of mitochondria*

Rat liver mitochondria were prepared as in [12], except that the tissue was homogenized in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl (pH 7.2), and 0.1 mM *NNN'N'*-ethylendiamintetraacetate, and the mitochondria were washed in a medium without *NNN'N'*-ethylendiamintetraacetate.

The protein content of the mitochondria was determined by a modification of the biuret procedure [13] using plasma albumin (Sigma Chemical Co., St Louis, MO) as standard.

2.2. *Measurement of Ca^{2+} fluxes using a Ca^{2+}-selective electrode*

Changes in the extramitochondrial concentration of Ca^{2+} were measured by means of a Ca^{2+}-selective electrode developed in [14]. The electrode response was amplified by Philips PW 949100 pH-meter, and displayed on a Perkin Elmer model 56 recorder (Norwalk, CT).

The calibration was done by adding known volumes of standard Ca^{2+} solutions (titrisol, Merck, Darmstadt) to the medium and plotting the ΔmV against ΔCa^{2+} (total) according to a linear scale.

Each incubation contained a standard medium composed of 200 mM sucrose, 20 mM Tris–*N*-(2 hydroxyethyl)-piperazine-*N'*2-ethane sulphonate (pH 7.2), 5 μg rotenone, 7.5 mM $MgCl_2$, 0.75 mM Tris–phosphate (pH 7.2), 6 mM Tris–succinate (pH 7.2) and 30 nmol Ca^{2+}/mg protein. The final volume was 3.5 ml, at 25°C.

2.3. *Measurement of O_2-consumption*

This was carried out at 25°C with a Clark-type O_2-electrode (Gilson, Oxygraph KIC, Villiers-le-Bel). The standard incubation medium was that described for the measurement of Ca^{2+} fluxes. The final volume was 0.7 ml.

2.4. *Measurement of membrane potential*

The electrical potential difference across the inner mitochondrial membrane was determined from the distribution of ^{86}Rb across the membrane in presence of valinomycin.

Mitochondria (2 mg/ml) were incubated at 25°C in a medium containing 200 mM sucrose, 20 mM Tris–*N*-(2 hydroxyethyl) piperazine-*N'*-2-ethanesulphonate (pH 7.2), rotenone (12 μg) 7.5 mM $MgCl_2$, 0.75 mM Tris–phosphate (pH 7.2) 20 mM RbCl, valinomycin (0.05 μg/ml).

Ca^{2+} (30 nmol/mg), 6 mM Tris–succinate (pH 7.2), $^{86}RbCl$ (1.6 nCi/ml) or $^{3}H_2O$ (0.3 μCi/ml). The final volume was 8 ml. At intervals, 1 ml samples were centrifuged for 5 min with a Eppendorf centrifuge 3200 (Dr Vaudaux and Co., Binningen). The pellets and supernatants were acidified with 15% $HClO_4$, centrifuged. Samples of the extracts were counted in 10 ml scintillation medium composed of 6 g butyl 2 (4'-*t*-butylphenyl)-5(-4''-biphenylyl)-1,3,4 oxadiazole and 80 g naphthalene in a mixture of 0.6 l toluene and 0.4 l methoxyethanol. The extramitochondrial and intramitochondrial volumes were determined by a similar process with [^{14}C]sucrose (50 nCi/ml) and $^{3}H_2O$ (0.32 μCi/ml).

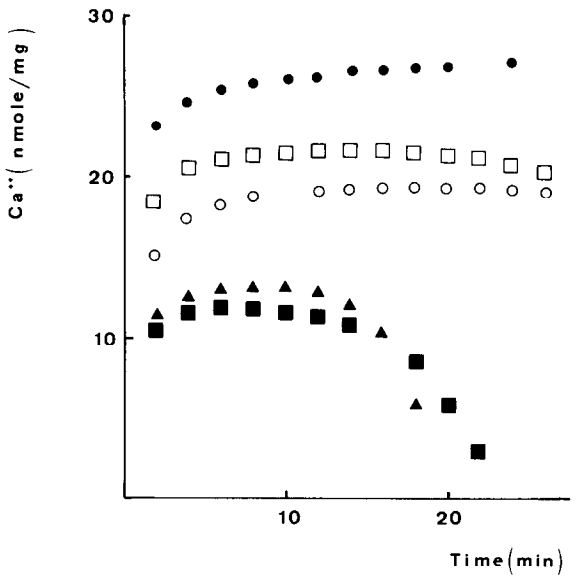

Fig.1. The release of Ca^{2+} from liver mitochondria by phosphoenolpyruvate. The reaction was started by adding 2 mg mitochondria/ml to the standard medium (see section 2), which contained the following: (•) no further addition; (▲) 2.9 mM PEP; (□) 400 μM atractylate + 2.9 mM PEP; (○) 1 mM ATP + 2.9 mM PEP; (■) 10 mM benzene 1,2,3-tricarboxylate + 2.9 mM PEP.

3. Results and discussion

Figure 1 reports the effects of PEP on the accumulation and retention of Ca^{2+} by rat liver mitochondria. In the absence of PEP, the mitochondria accumulate almost all the Ca^{2+} available (about 30 nmol/mg protein) and retain it for at least 30 min. With 3 mM PEP present, the mitochondria accumulate less Ca^{2+} and the Ca^{2+} that is taken up is released within 25 min. PEP is transported by both the adenine nucleotide and tricarboxylate carriers of liver mitochondria [15,16]. Figure 1 shows that inhibition of the adenine nucleotide carrier by atractylate [17,18] or ATP prevent the ability of PEP to induce Ca^{2+} release, whereas inhibition of the tricarboxylate carrier with benzene-1,2,3-tricarboxylate [16] does not. These data indicate that the action of PEP is not dependant on its penetration into the mitochondrial matrix per se, but that specific transport by the adenine nucleotide carrier is required.

This conclusion supports that made in [9], that removal of endogenous ATP (by exchange with PEP) is the essential factor in the induction of Ca^{2+} release. In this case, intramitochondrial ATP is presumed to facilitate retention of Ca^{2+} by promoting the precipitation of calcium phosphate in the mitochondrial matrix, as reported in [19].

However, whilst the degree of intramitochondrial precipitation of Ca^{2+} may be an important factor in its retention by mitochondria, it is equally important to establish whether or not the minimisation of precipitation by PEP is sufficient in itself to permit release, or whether this phenomenon entails other effects on mitochondrial function. Figure 2 shows that the addition of PEP to mitochondria respiring in the presence of Ca^{2+} causes a progressive increase in the rate of respiration. In addition, when PEP uncouples respiration, the electrical potential across the inner membrane ($\Delta\psi$) decreases to about 35 mV after 30 min, compared to a control value of about 120 mV which is maintained in the absence of added PEP (fig.3).

The dissipation of respiratory energy under these

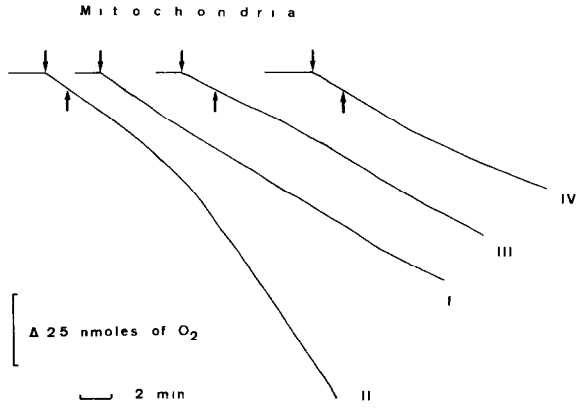

Fig.2. The effect of phosphoenolpyruvate on the respiration of liver mitochondria. The reaction was started by adding 0.3 mg mitochondria to the standard medium (see section 2). The following additions were made (as indicated by the arrows): (I) none; (II) 2.9 mM PEP; (III) 2.9 mM PEP + 2.5 nmol RR/mg protein; (IV) 2.9 mM PEP + 1 mM EGTA.

conditions is not due to PEP per se, since no uncoupling or decrease of $\Delta\psi$ is seen in the presence of EGTA or ruthenium red. EGTA complexes extramitochondrial Ca^{2+}, whereas ruthenium red is a specific inhibitor of the system that catalyses Ca^{2+} influx across the inner membrane [20]. Energy dissipation appears to require intramitochondrial Ca^{2+} in addition to PEP.

One possibility is that PEP induces efflux of Ca^{2+} via a system distinct from that responsible for influx, so that recycling of Ca^{2+} via the PEP-induced efflux and reuptake on the natural influx carrier is responsible for the energy dissipation. This was investigated by determining the rate of PEP-induced efflux when uptake is inhibited by ruthenium red (RR) (fig.4). As reported [5], addition of ruthenium red causes net Ca^{2+} release in liver mitochondria, and, in addition, PEP does augment this process slightly. However, the rate of 'extra' Ca^{2+} release induced by PEP in the presence of RR (0.53 nmol. mg protein^{-1}.min^{-1}) is extremely slow, and permitting Ca^{2+} recycling at the same rate, would only account for a respiration increase of 0.066 nmol O_2.mg protein^{-1}.min^{-1}, which is much less than the observed respiring increment

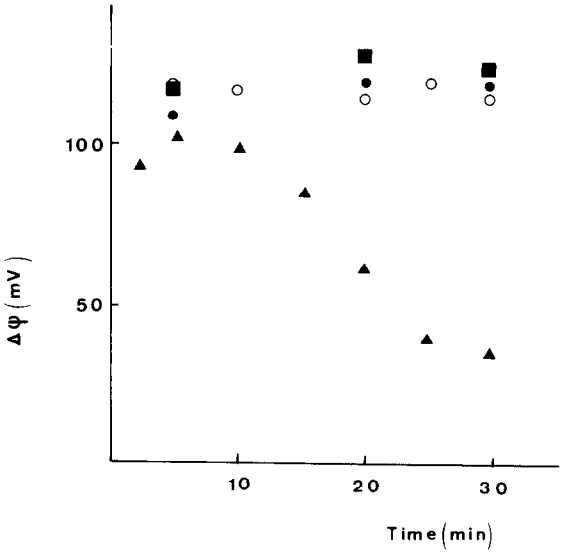

Fig.3. The effect of phosphoenolpyruvate on the electrical potential across the inner mitochondrial membrane. The membrane potentials were measured as in section 2. Symbols: (●) no PEP; (▲) 2.9 mM PEP; (■) 2.9 mM PEP + 2.5 nmol RR/mg protein; (○) 2.9 mM PEP + 1 mM EGTA.

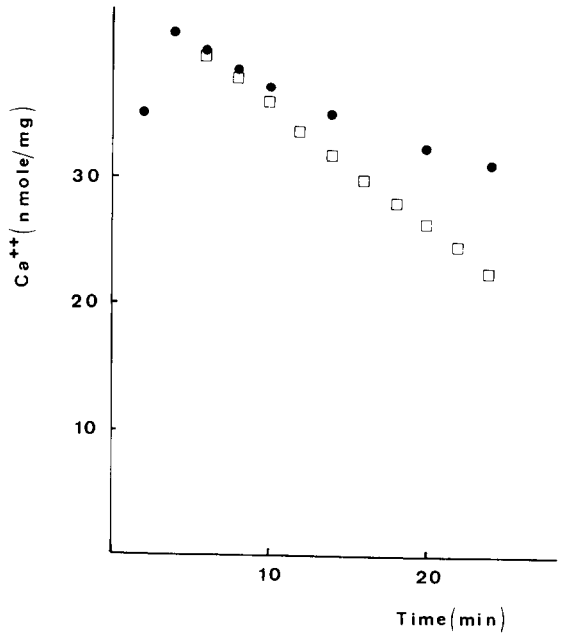

Fig.4. The efflux of Ca^{2+} in the presence of RR and PEP. The reaction was started by adding 2 mg mitochondria/ml to the standard medium (see section 2). Curve a (●) addition of 2.5 nmol RR/mg protein after 6 min. Curve b (□) addition of 2.5 nmol RR/mg protein and 2.9 mM PEP after 6 min.

of 67.5 nmol O_2. mg protein^{-1}.min^{-1} induced by PEP (fig.2).

The above data suggest therefore that the PEP-induced efflux of Ca^{2+} occurs essentially via reversal of the energy-linked uptake process. This requires a decrease in the driving force for Ca^{2+} influx via this system, and as shown, the presumed driving force, $\Delta\psi$, does decrease to very low values. The reason for the fall in $\Delta\psi$ is presumably the uncoupling of respiration.

The factors responsible for respiratory uncoupling are unknown, but one may speculate that an increase in intramitochondrial ionized Ca^{2+}, due to its impeded precipitation as the phosphate salt may have an adverse effect on the inner membrane although at the moment no suggestions can be made as to the nature of this hypothetical effect. In any event, the important point is that Ca^{2+} release by PEP appears to depend upon such deletereous effects on mitochondrial functions. Hence, it is concluded that the release of Ca^{2+} by PEP probably does not reflect the physiological mechanism by which Ca^{2+} efflux is achieved.

On the other hand, PEP due to its ability to 'free' Ca^{2+} inside documented in this paper, may facilitate the operation of the natural release mechanism. There is evidence that Ca^{2+} release from liver mitochondria may proceed via a discrete efflux route, separate from the influx pathway.

Acknowledgements

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