PHOSPHATE-INDEPENDENT CALCIUM EFFLUX FROM LIVER MITOCHONDRIA

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1. Introduction

Liver mitochondria possess independent pathways for the uptake and efflux of Ca^{2+} (reviews [1,2]). The mechanism of the efflux pathway is unclear; net Ca^{2+} efflux leads to an overall exchange of Ca^{2+} for 2 H^+ across the mitochondrial membrane [3], although there is no evidence that this reflects a strict exchange between protons and Ca^{2+} at the efflux carrier since other charge-compensating proton movements may occur [1]. It has been suggested [4–6] that the liver efflux pathway operates as a Ca^{2+}:P_i symport, based on the observation that P_i induces a time-dependent net efflux of Ca^{2+} under some conditions. A possible complication in studies of P_i-induced Ca^{2+} efflux is that in incomplete incubation media, lacking Mg^{2+} and adenine nucleotides, the combination of Ca^{2+} and P_i can result in a loss of matrix contents, including Mg^{2+}, adenine nucleotides and K^+ [6,7] leading to a collapse of $\Delta\psi$ and an artifactual release of Ca^{2+} [7,8].

Here, we re-examine the role of P_i in Ca^{2+} efflux by incubating mitochondria in the presence of Mg^{2+} and adenine nucleotides and monitoring ruthenium red-induced efflux and $\Delta\psi$ simultaneously. We observe that under these stable conditions P_i strongly inhibits the efflux pathway. It is suggested that P_i acts by lowering the free matrix Ca^{2+} below the concentration required to saturate the efflux pathway, but that since maximal efflux is observed in P_i-depleted mitochondria, there is no evidence for an obligatory co-transport of P_i with Ca^{2+}.

Abbreviations: $\Delta\psi$, mitochondrial membrane potential; pCa_o^{2+}, the negative log of the extra-mitochondrial free Ca^{2+} concentration

2. Materials and methods

Rat liver mitochondria were prepared as in [9]. Mitochondrial protein was determined by the biuret method [10]. $^{45}Ca^{2+}$ accumulation, pCa_o^{2+} and $\Delta\psi$ were determined simultaneously by incubating mitochondria (1.5 mg protein/ml incubation) at 37°C and pH 7.0 in a medium containing 100 mM NaCl, 25 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino)-ethane sulphonate (sodium salt), 16 μM albumin (bovine, fraction V), 2 mM succinate (sodium salt), 1 μM rotenone, 2 mM nitrilotriacetate (sodium salt), 1 mM $MgCl_2$, 1 μM [^{3}H]methyltriphenylphosphonium bromide (0.16 μCi/ml) and various concentrations of $^{45}Ca^{2+}$ (0.05 μCi/ml). The volume of the matrix was determined as in [9] and was found to be 0.74 μl/mg protein.

At defined times, 300 μl aliquots of the incubation were withdrawn and layered onto 250 μl of a mixture of 60% (v/v) Dow Corning 550 silicone fluid and 40% dinonylphthalate contained within an Eppendorf tube and centrifuged for 60 s. A portion of the supernatant was taken for counting $^{45}Ca^{2+}$ and ^{3}H. Mitochondrial uptake of the isotopes was calculated by comparison with the counts in an identical volume of uncentrifuged incubation.

The initial Ca^{2+} content of the medium and endogenous mitochondrial Ca^{2+} were determined in acid extracts by a Corning-Eel Model 240 atomic absorption spectrophotometer. pCa_o^{2+} was calculated from the supernatant [Ca^{2+}] after allowing for chelation by the nitrilotriacetate [11]. To deplete mitochondria of endogenous P_i, 0.2 mM ADP, 1 mM glucose and 0.75 units/ml of hexokinase were added to the basic incubation. For the determination of P_i, 0.75 ml aliquots of the mitochondrial suspension were deproteinated with 20% (final conc. v/v) trichloroacetic acid and P_i was analyzed by the method in [12].

3. Results and discussion

Fig.1 shows the effect of varying $[P_i]$ upon the rate of ruthenium red-induced net Ca^{2+} efflux from liver mitochondria incubated in the presence of ATP, oligomycin and Mg^{2+}. As $[P_i]$ is lowered from 3.3 mM to endogenous levels (60 μM) there is a steady increase in the Ca^{2+} efflux rate from 1–8 nmol Ca^{2+} . min^{-1} . mg $protein^{-1}$. The pCa_o^{2+} maintained by the mitochondria prior to ruthenium red addition was marginally affected by the activation of the efflux pathway, decreasing from 6.24 in the presence of 3.3 mM P_i to 6.14 in the presence of endogenous P_i. This was however only sufficient to alter the matrix Ca^{2+} content by 2 nmol/mg protein, and a mean value is shown in fig.1 for matrix Ca^{2+} before addition of ruthenium red. $\Delta\psi$ was maintained at a high value for all $[P_i]$ and times, the extreme range for all 56 determinations being only 163–174 mV. The high potential in the absence of added P_i indicates that endogenous P_i is sufficient to compensate the uptake of up to 60 nmol Ca^{2+}/mg protein, as reported in [11].

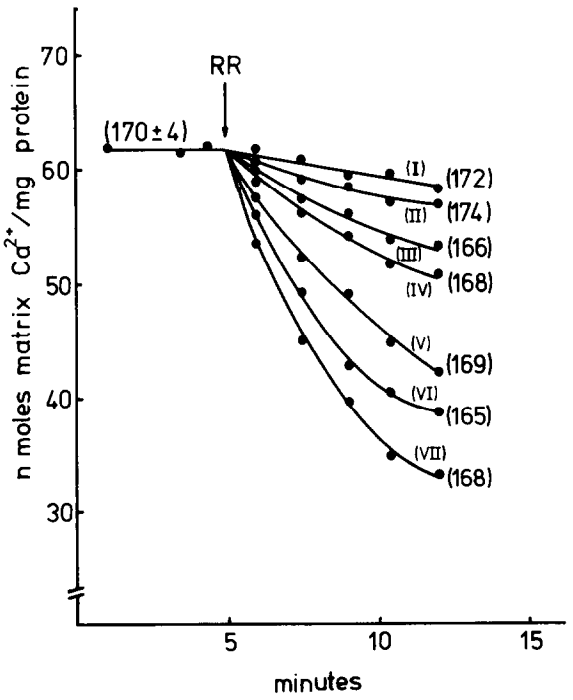

Fig.1. The effect of P_i on Ca^{2+} efflux induced by ruthenium red. Mitochondria were incubated in the basic medium with the additions of 1 μg oligomycin/ml, 0.2 mM ATP and 83 nmol Ca^{2+}/mg protein; 0.9 μM ruthenium red (RR) was added where indicated. $[P_i]$ including endogenous P_i was: (I) 3.3 mM; (II) 2 mM; (III) 1 mM; (IV) 0.66 mM; (V) 0.36 mM; (VI) 0.16 mM; (VII) 0.06 mM. Values in parentheses are $\Delta\psi$ (mV) determined at 4 min or 12 min.

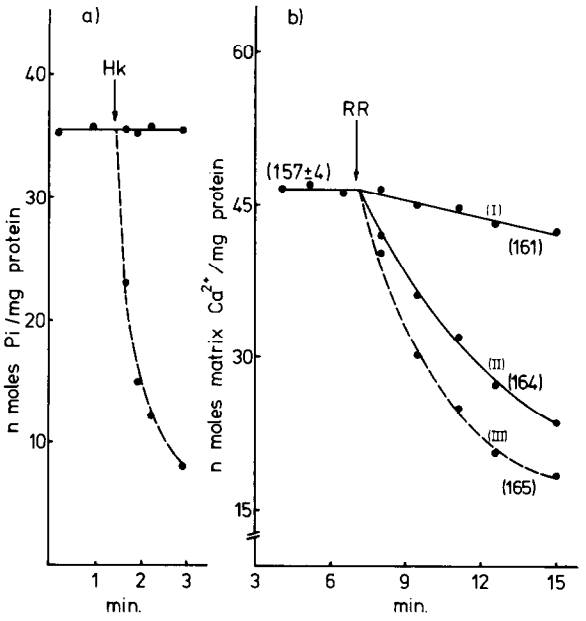

Fig.2. The effect of P_i depletion on Ca^{2+} efflux induced by ruthenium red. Mitochondria were incubated in the basic medium with the additions of 1 mM glucose, 0.2 mM ADP, 5 mM acetate (sodium salt) and 26 nmol Ca^{2+}/mg protein. In (a) the decrease in endogenous P_i following addition of hexokinase is determined. In the same incubation, at 3.5 min an additional 35 nmol Ca^{2+}/mg protein was added (b) and Ca^{2+} content was determined, 0.9 μM ruthenium red (RR) was added at 7 min; (III) follows the efflux of Ca^{2+} from these P_i depleted mitochondria; (I) no hexokinase added, 2 mM P_i present from the start; (II) no hexokinase added, endogenous P_i (36 nmol/mg protein). Values in parentheses are $\Delta\psi$ (mV) determined at 6 min and 15 min.

In order to deplete endogenous P_i, mitochondria were preincubated in the presence of ADP, glucose and hexokinase prior to ruthenium red addition, thus allowing endogenous P_i to phosphorylate ADP and become trapped as glucose 6-phosphate. Fig.2a shows the time-course of P_i depletion by this technique, while fig.2b demonstrates that ruthenium red-induced efflux is still further activated by this technique. In these experiments 5 mM acetate was present throughout; as before $\Delta\psi$ was maintained at >160 mV.

The conclusion from these experiments is that when liver mitochondria are incubated under stable conditions, P_i inhibits rather that activates net Ca^{2+} efflux. In addition, since maximal efflux rates are observed in mitochondria which have been extensively depleted

of P_i this makes the possibility that the efflux pathway operates by Ca^{2+}:P_i co-transport extremely unlikely.

There has been one report that P_i-induced Ca^{2+} efflux from liver mitochondria incubated in the absence of Mg^{2+} or adenine nucleotides occurs by a mechanism other than membrane potential collapse [13]. However, $\Delta\psi$ and Ca^{2+} flux were determined in separate experiments under apparently different conditions (valinomycin being present for the $\Delta\psi$ determination and arsenazo III for the spectrophotometric Ca^{2+} determination). Since the time of onset of P_i-induced Ca^{2+} release is highly variable in replicate experiments (e.g., fig.3,6 in [13]) it is not possible by separate experiments to prove the temporal relationships of Ca^{2+} release, P_i release and $\Delta\psi$ collapse. Significantly, when $\Delta\psi$ and Ca^{2+} transport are monitored in the same incubation during the acetoacetate potentiation of P_i-induced Ca^{2+} release, collapse of potential and release of Ca^{2+} occur with exact simultaneity [8].

The slow net efflux of Ca^{2+} in the presence of P_i which is observed here is not due to a limitation in the rate at which the matrix $Ca \cdot P_i$ complex can dissociate, since the addition of 0.25 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to lower $\Delta\psi$ induces a net efflux in the absence of ruthenium red which is in excess of 50 nmol $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ (not shown). The alternative possibility is that in the presence of P_i the free matrix $[Ca^{2+}]$ is held below the level required to saturate the efflux pathway, and this is currently being investigated.

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