ON THE NATURE OF P_i-INDUCED, Mg^{2+}-PREVENTED Ca^{2+} RELEASE IN RAT LIVER MITOCHONDRIA

Paolo BERNARDI and Daniela PIETROBON

CNR Unit for the Study of Physiology of Mitochondria and Institute of General Pathology, Via Loredan 16, 35100 Padova, Italy

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1. Introduction

The pathway and role of mitochondrial Ca^{2+} efflux has assumed a major importance (review [1–3]). Although it is generally accepted that at steady state Ca^{2+} undergoes a continuous, slow recycling across the inner mitochondrial membrane, due to the simultaneous operation of the influx and efflux pathways [4], the nature and regulation of the efflux pathway are matters of debate [1–3].

Since 1964 it has been known that inorganic phosphate (P_i) releases Ca^{2+} from liver mitochondria [5]. The Ca^{2+} release is irreversible and accompanied by indefinite stimulation of respiration [5]. Although these phenomena have been attributed to increased Ca^{2+} cycling, and therefore to stimulation by P_i of a specific Ca^{2+} efflux pathway [6–8], direct evidence for such a specific pathway and for real Ca^{2+} cycling has never been provided.

Here, we aim to clarify the nature of P_i-induced, Mg^{2+}-prevented Ca^{2+} release and of the related respiratory stimulation. We show that, in the absence of Mg^{2+} and BSA, phosphate addition to Ca^{2+}-preloaded mitochondria causes a sharp decrease of ΔpH. Since $\Delta\psi$ tends to decline as well, the H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) decreases dramatically. The nature of Ca^{2+} release induced by P_i in the absence of Mg^{2+} appears therefore to be due to reversal of the uniport carrier, consequent to decrease of $\Delta\psi$ rather than to activation of an independent efflux pathway. Accordingly, the indefinite stimulation of respiration is not due to increased Ca^{2+} cycling but to increased H^+ recycling across the inner membrane.

Abbreviations: EGTA, (ethylene-bis(oxoethylenenitrilo))tetraacetic acid; BSA, bovine serum albumin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, 4-morpholinepropane sulfonic acid; HEDTA, *N'*-(2-hydroxyethyl)ethylene-diamine-*N,N,N'*-triacetic acid; $TPMP^+$, triphenylmethylphosphonium ion; DMO, 5,5-dimethyl-2,4-oxazolidinedione; pCa_o, $-\log [Ca^{2+}]$ outside the mitochondrial compartment; $\Delta\psi$, membrane potential

2. Materials and methods

Rat liver mitochondria were prepared as in [9] in 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4) and 0.1 mM EGTA. The final washing was carried out in an EGTA-free medium and mitochondrial protein was assayed with the biuret method, using BSA as a standard. The incubation media are specified in the figure legends.

Oxygen consumption was monitored with a Clark oxygen electrode (Yellow Springs Instruments, OH) in a magnetically stirred, thermo-equilibrated vessel.

Ca^{2+} movements were monitored with a Ca^{2+}-selective electrode (W. Möller, Zürich) in a waterjacket-thermostatted vessel equipped with magnetic stirring. The Ca^{2+}-electrode was routinely calibrated with Ca^{2+}–HEDTA buffers containing 50 mM HEDTA, 2 mM $MgCl_2$, Tris–Mops buffer (pH 7.0) and $CaCl_2$ to give the desired free $[Ca^{2+}]$ (apparent stability constants were taken as 2.57×10^5 (Ca^{2+}) and 1.12×10^3 (Mg^{2+}) at pH 7.0 [4]). The value of $[Ca\text{–}HEDTA_{total}]$ was taken as being equal to the total $[Ca^{2+}]$, since $[Ca^{2+}] \ll [Ca\text{–}HEDTA_{total}]$.

In the isotope experiments mitochondria were incubated under the specified conditions with [^{14}C]-$TPMP^+$ (0.02 μCi/ml, corresponding to final conc. 20 μM $TPMP^+$) or with [^{14}C]DMO (0.07 μCi/ml). After the specified time the suspensions were centrifuged at 30 000 × *g* in a Sorvall RC2B refrigerated

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supercentrifuge for 5 min. The clear supernatants were decanted, the tube wall blotted dry, and the pellets dissolved by treatment with 0.2 ml of a 1 mM Na-EDTA solution containing 0.1% (w/w) NaCl and 0.9% (w/w) sodium deoxycholate, at room temperature. The dissolved pellets and 100 μl aliquots of the supernatants were added to 2 ml of Packard Insta-Gel scintillation fluid. Mitochondrial volumes were calculated on parallel samples containing ^3H_2O (1 μCi/ml) and [^{14}C]sucrose (0.2 μCi/ml). The radioactivity was assayed with a Packard Tri Carb 300 C liquid scintillation spectrometer and the dpm were calculated using an external standard method and calibration curves to correct for quenching [10].

Membrane potential was calculated on [^{14}C]TPMP$^+$ distribution across the inner mitochondrial membrane. A correction for passive TPMP$^+$ binding was introduced, measuring the amount of radioactivity present in mitochondria treated with FCCP + valinomycin + antimycin A in the presence 0–0.5 mM unlabeled TPMP$^+$. Since the radioactivity found in the pellets did not decrease at still higher concentrations of TPMP$^+$, the difference between the dpm at 0.5 mM and 0 TPMP$^+$ is taken as passive binding. This correction rests on the assumption that the TPMP$^+$ binding is the same in energized and de-energized mitochondria. The activity coefficient assumed for intramitochondrial TPMP$^+$ was 1. Obviously, both assumptions are only approximately correct, and the values of $\Delta\psi$ could be overestimated. The membrane potential was calculated from the Nernst equation.

ΔpH was measured on [^{14}C]DMO distribution across the inner membrane, according to [11].

3. Results

Fig.1A shows the effects of P_i on steady state Ca^{2+} distribution in mitochondria incubated in the absence of Mg^{2+} and BSA. After the uptake of ~15 nmol Ca^{2+}/mg protein, addition of 2 mM P_i caused only a very slight increase of Ca^{2+} uptake. When a further 80 μM Ca^{2+} was added, Ca^{2+} uptake was followed by a sudden and irreversible Ca^{2+} release. Thus, in the absence of Mg^{2+}, P_i does not significantly alter the steady state Ca^{2+} distribution while it induces Ca^{2+} efflux at high Ca^{2+} loads. Fig.1B shows that, in the presence of Mg^{2+} and BSA, the steady state extramitochondrial pCa_o is poised at lower levels, in accord with [4,12]. Addition of P_i then caused a slow Ca^{2+} efflux until a higher steady state pCa_o was attained.

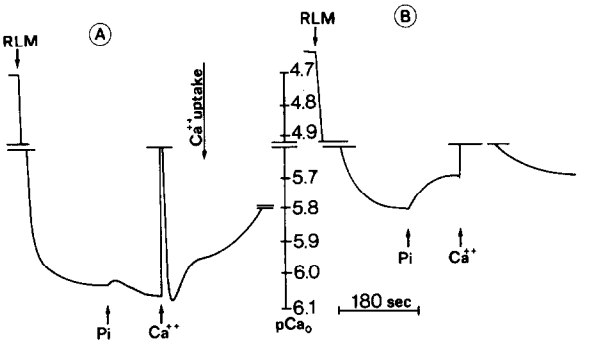

Fig.1. Prevention by Mg^{2+} and BSA of P_i-induced Ca^{2+} release. (A) Incubation medium contained 0.14 M sucrose, 40 mM choline chloride, 10 mM Tris–Mops (pH 7.0), 5 mM succinate–Tris, 16 μM Ca^{2+}. In (B) 2 mM $MgCl_2$ and 1 mg/ml BSA were added. Final volume 5 ml, 30°C. When indicated 5 mg mitochondria (RLM), 2 mM P_i and 80 μM Ca^{2+}.

The specific features of this novel P_i-stimulated Ca^{2+} efflux are analyzed in detail in [13]. It is important here to stress that in the presence of BSA and Mg^{2+} a further 80 μM Ca^{2+} pulse may be taken up, and that the same steady state Ca^{2+} distribution preceeding the first Ca^{2+} addition is reached and maintained until anaerobiosis (not shown). Thus the process of complete and irreversible Ca^{2+} release caused by P_i is abolished by BSA and Mg^{2+}.

Fig.2 analyzes the nature of the respiratory stimu-

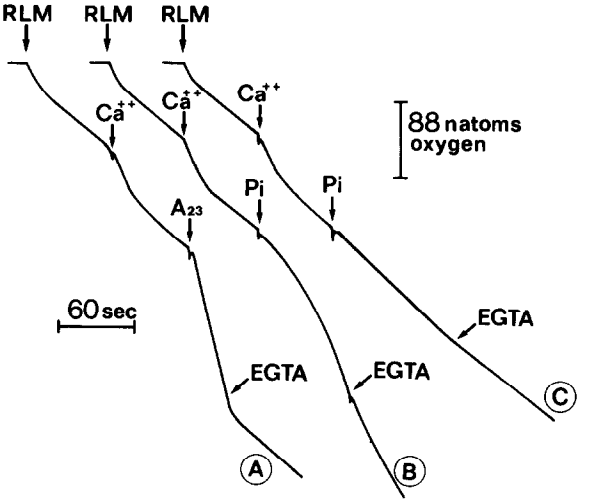

Fig.2. Effect of BSA, Mg^{2+} and EGTA on respiratory stimulation following Ca^{2+} and P_i uptake. The incubation medium was the same as fig.1A plus 5 mM acetate–Tris. In (C) 2 mM $MgCl_2$ and 1 mg/ml BSA were added. Final volume 2 ml, 30°C. When indicated, 4 mg mitochondria (RLM), 90 μM Ca^{2+}, 2 μg A23187 (A 23), 2 mM P_i and 0.25 EGTA.

lation due to Ca^{2+} plus P_i. Trace 2A shows that addition of the electroneutral Ca^{2+} ionophore A23187 after a Ca^{2+} pulse caused an immediate increase of respiration, due to continuous Ca^{2+} recycling across the inner membrane [14]. This is confirmed by the fact that addition of EGTA brought the respiration to the state 4 level preceeding the addition of the ionophore. In trace 2B, after the Ca^{2+} pulse, 2 mM P_i was added instead of A23187. Respiration was gradually stimulated after a lag phase [5,7,8] while addition of EGTA had only a very slight effect. Trace 2C indicates that in the presence of Mg^{2+} and BSA, P_i had negligible effects on mitochondrial respiration, and that EGTA again slightly depressed state 4 respiration.

Table 1 analyzes the values of $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_{H^+}$ in Ca^{2+}-loaded mitochondria, and the effects of P_i on these values in the presence or absence of Mg^{2+} plus BSA.

In the absence of P_i, Ca^{2+}-loaded mitochondria maintained a ΔpH of 89 mV and a $\Delta\psi$ of 121 mV, with a total $\Delta\tilde{\mu}_{H^+}$ of 210 mV. ΔpH was decreased to very low values after addition of P_i. $\Delta\psi$ on the other hand increased markedly in the presence but not in the absence of Mg^{2+} + BSA, almost completely compensating the drop in ΔpH in the former but not in the latter case. Furthermore $\Delta\psi$ was kept at a higher level after addition of EGTA (101 mV) but not after addition of ruthenium red. Table 1 thus shows that partial restoration of $\Delta\tilde{\mu}_{H^+}$ requires removal of Ca^{2+} from the medium and not simple abolition of Ca^{2+} cycling by ruthenium red.

Table 1
$\Delta\psi$ and ΔpH following Ca^{2+} and P_i uptake

Exp.	Addition(s) to basic medium	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_{H^+}$ (mV)
1	None	121	89	210
2	2 mM P_i	47	0	47
3	2 mM P_i, 1 mg/ml BSA, 2 mM Mg^{2+}	164	10	174
4	2 mM P_i, 2 μM ruthenium red	56	4	60
5	2 mM P_i, 0.5 mM EGTA	101	10	111

The incubation medium was as in fig.1A plus 20 μM [^{14}C]-TPMP$^+$ ($\Delta\psi$ measurements) or carrier-free [^{14}C]DMO (ΔpH measurements) in parallel samples. Total calcium was 80 nmol/mg protein. Further additions to the medium were as indicated. The experiments were started by the addition of 5 mg mitochondria to 2 ml final vol. at room temperature. In exp. 1–3 $\Delta\psi$ and ΔpH were determined after 4 min incubation. In exp. 4,5, ruthenium red or EGTA were added after 4 min incubation, and $\Delta\psi$ and ΔpH were determined after a further 1 min. Matrix volumes were calculated on parallel samples containing ^3H_2O and [^{14}C]sucrose. For further explanation see section 2

4. Discussion

Since the early fifties it has been known that addition of P_i results in a large-amplitude mitochondrial swelling which is reversed by ATP + Mg^{2+} (review [15,16]). The P_i-induced swelling is markedly enhanced by the simultaneous uptake of Ca^{2+}, and is accompanied by an increased permeability of the mitochondrial membrane and by subsequent release of matrix constituents [17,18]. In 1964 it was shown that P_i releases Ca^{2+} from mitochondria [5]. Ca^{2+} release required a threshold concentration of Ca^{2+} or P_i, and was accompanied by an indefinite stimulation of the respiration, which was attributed to increased Ca^{2+} cycling across the inner membrane [5]. This view has been re-evaluated by different groups [6–8], and it has been tacitly assumed that stimulation of respiration after completion of Ca^{2+} uptake is a reflection of increased Ca^{2+} cycling [6–8]. Due to this view, it has been concluded that Sr^{2+}, at variance from Ca^{2+}, is unable to cycle across the mitochondrial membrane from the observation that addition of P_i after Sr^{2+} uptake does not result in stimulation of respiration [7].

Our data indicate that the P_i-induced, Mg^{2+}- and BSA-prevented Ca^{2+} efflux is due to a reversal of Ca^{2+} transport through the uniport carrier following a decrease of the transmembrane electrical potential. Furthermore, the related increase in respiration is mainly due to an increased H^+ recycling and not to a Ca^{2+} recycling. This is supported by four lines of evidence:

(i) The P_i-induced Ca^{2+} efflux occurring in the absence of Mg^{2+} (fig.1A) and the related increase of respiration (fig.2) are prevented by Mg^{2+} and BSA, which are agents well known to protect against membrane damage [19];

(ii) The P_i-induced Ca^{2+} efflux does not lead to a new set point for Ca^{2+} distribution, as required by the establishment of a kinetic steady state between Ca^{2+} influx and efflux processes, but rather to a complete Ca^{2+} release;

(iii) The stimulation of respiration induced by Ca^{2+} plus P_i is almost unaffected by the addition of EGTA, which is expected to stop Ca^{2+} cycling, as it occurs in the case of the ionophore A23187 (fig.2A);

(iv) The data of table 1 indicate that in the absence of P_i, Ca^{2+} uptake results in establishment of a large ΔpH.

In the absence of Mg^{2+} and BSA, addition of P_i causes a collapse of ΔpH without parallel increase of $\Delta\psi$. The lack of $\Delta\psi$ rise, to an extent complementary to the depression of ΔpH, as required to maintain an unaltered $\Delta\tilde{\mu}_{H^+}$, cannot be ascribed to energy-dissipating Ca^{2+} recycling, since ruthenium red does not cause increase of $\Delta\psi$. The partial $\Delta\psi$ recovery induced by EGTA can be explained by inhibition of Ca^{2+}-stimulated phospholipases [20]. Thus the finding of a 5 mV decrease of the membrane potential upon addition of 1.6 mM P_i, with concomitant Ca^{2+} efflux [6], means in fact a presumable 60 mV decrease of $\Delta\tilde{\mu}_{H^+}$. Furthermore, when $\Delta\psi$ is below the critical value of ~125 mV, Ca^{2+} distribution is at electrochemical equilibrium, and any decrease of $\Delta\psi$ allows Ca^{2+} efflux to occur via a reversal of the uniport Ca^{2+} carrier [4].

In conclusion we suggest that distinction between a Ca^{2+} efflux occurring through a reversal of the Ca^{2+} uniporter or through activation of an independent efflux pathway should be achieved by a number of different criteria, among which the attainment of a new steady state and the determination of both ΔpH and $\Delta\psi$ play a critical role. On the basis of these criteria evidence for a $\Delta\psi$-modulated pathway for Ca^{2+} efflux, stimulated by P_i and requiring Mg^{2+}, is presented in [13].

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