THE ELECTRIC CHARGE STOICHIOMETRY OF CALCIUM INFLUX IN RAT LIVER MITOCHONDRIA AND THE EFFECT OF INORGANIC PHOSPHATE

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1. Introduction

There is considerable evidence that calcium is translocated across the inner mitochondrial membrane by an electrophoretic process responding to an electrochemical gradient, negative inside, generated either by respiration, by ATP hydrolysis or by passive ion-efflux produced by specific ionophores. It was generally agreed that two positive charges were carried per calcium ion transported into the mitochondrion [1–10].

However, a different mechanism has been proposed in which calcium-uptake occurs via a carrier that catalyses a calcium–phosphate symport [11,12]. According to this the positive charges of Ca^{2+} are partially compensated by the negative charge of phosphate so that each Ca^{2+} enters with an effective net charge of 1.

The electric charge stoichiometry of calcium transport is of fundamental importance for the understanding of both the mitochondrial energy-coupling mechanism and the regulatory system by which the mitochondria control the cellular concentration of calcium.

We have carried out experiments using K^+-efflux induced by valinomycin as driving force for Ca^{2+}-uptake and measuring directly and simultaneously the movement of all relevant ions presumably involved in the process. We have found that 1 Ca^{2+} is translocated into the mitochondria in exchange for 2 K^+, and that no phosphate-uptake occurs during this process. Addition of phosphate or NEM (to block phosphate transport)-induced changes in the rate of calcium uptake but did not affect significantly the K^+/Ca^{2+} ratio.

2. Materials and methods

Rat liver mitochondria were isolated as in [13] in a medium consisting of 0.25 M sucrose, 5 mM Tris–MOPS (pH 7.2), 1 mM EGTA. They were washed 3 times, omitting the EGTA in the last 2 washings. The experiments were carried out at 30°C in the cell of a dual wavelength spectrophotometer made in the workshop of this Department; the cell was equipped with a magnetic stirrer. A Clark oxygen electrode, a K^+-sensitive electrode and a pH glass electrode were inserted into the cell through independent openings; the last two electrodes were connected to Philips pH-meters (model PW 9418), the outputs from which were fed to a 4 channel recorder.

Ca^{2+} movements were followed either spectrophotometrically or with a Ca^{2+}-sensitive electrode. In the first case arsenazo III was used as indicator fol-

Abbreviations: arsenazo III, 2-2'-(1-8 dihydroxy-3,6-bisulfo-2,7-naphtalene-bis azo) dibenzene arsonic acid; EGTA, ethylenglycol-bis-(aminoethyl) tetracetate; mersalyl, *O*-(3-hydroxy-mercuri-2-methoxypropyl) carbamoylphenoxy-acetate; MOPS, morpholinopropane–sulfonic acid; NEM, *N*-ethylmaleimide; TTFB, tetrachloro, trifluoromethoxy-benziimidazole

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lowing the $\Delta A_{590-570}$. The arsenazo III was neutralized with Tris-base and passed through a Chelex X-100 (Biorad) column before use. In the second case the Ca^{2+}-sensitive electrode was inserted in the cell instead of the oxygen electrode. The rate of mitochondrial swelling was monitored by recording the light scattering changes at 570 nm as in [14]. An internal calibration was carried out for each parameter in all experiments. The incubation standard medium contained: 0.12 M choline chloride, 0.5 mM Tris–MOPS (pH 7.2), 50 μM KCl, 7 mg protein in 3.5 ml final vol.

All parameters were recorded simultaneously with a 4 channel Rikadenki recorder; one channel was equipped with a time-sharing switching circuit which enabled 2 parameters to be recorded on one channel. Protein concentration was assayed by the biuret method using bovine serum albumin as standard [15]. P_i was assayed in the deproteinized extracts of mitochondria by the method in [16]. All chemicals were of analytical grade.

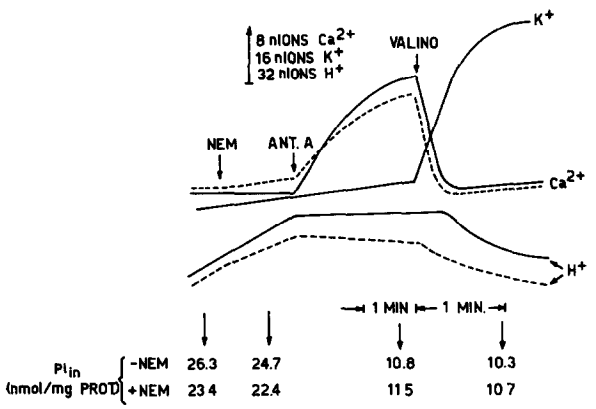

Fig.1. Effect of antimycin A, valinomycin and NEM on Ca^{2+}, K^+ and H^+ movements and phosphate content in rat liver mitochondria. Mitochondria were incubated for 2 min as indicated in section 2. Antimycin A (0.07–0.08 nmol/mg protein), valinomycin (2 pmol/mg protein) and NEM (40 nmol/mg protein) were added as indicated; rotenone (1 μM) was added together with antimycin. Dashed line, added NEM; full line, no added NEM. Aliquots of incubations medium were withdrawn at the arrows for phosphate determinations. Results are means of several experiments.

3. Results

Figure 1 shows the concentration changes of Ca^{2+}, K^+ and H^+ induced by addition of antimycin A and the subsequent addition of valinomycin. Antimycin was added at the minimum amount required to obtain an almost complete inhibition of oxygen consumption and a negligible uncoupling effect. Mitochondria used in these experiments were not loaded with additional calcium. Addition of the antimycin induced a release of Ca^{2+} at 4.6–6.3 ng ion/mg mitochondrial protein. This amount accounted for ~70% of the quantity of calcium released after addition of an uncoupling agent such as 2.5 μM TTFB.

When valinomycin was added at 1–2 pmol/mg protein, Ca^{2+} was transported into the mitochondria and there was no significant pH change; only when almost all Ca^{2+} was taken up was H^+-uptake observed. This H^+-uptake was accompanied by K^+-release. (The valinomycin concentration was of crucial importance: < 1 pmol/mg protein did not induce the total calcium uptake, whereas high amounts caused an immediate H^+-uptake simultaneously with that of Ca^{2+} and a very high rate of ion movement which rendered the calculation of the K^+/Ca^{2+} ratio difficult.)

Addition of NEM promoted a slight release of Ca^{2+}, together with a slight diminution of the rate of pH-decrease (see fig.1). The rate and the total amount of Ca^{2+} released after addition of antimycin A was less in the presence of NEM whereas the rate of valinomycin-induced Ca^{2+}-uptake was about the same. There was a release of phosphate from the mitochondria after addition of antimycin either in the presence or in the absence of NEM, confirming that phosphate release is insensitive to NEM probably due to an asymmetrical

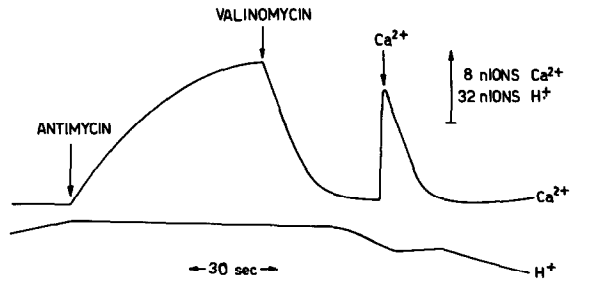

Fig.2. Effect of Ca^{2+}-addition on the pH-change. Experimental conditions as in fig.1. 5 ng ion Ca^{2+}/mg protein were added at the arrow.

Table 1
Initial rates of K^+-release and Ca^{2+}-uptake after addition of valinomycin

Additions	Initial rate of Ca^{2+}-uptake (ng ions .min^{-1} .mg $protein^{-1}$)	Initial rate of K^+-release (ng ions .min^{-1} .mg $protein^{-1}$)	K^+/Ca^{2+} ratio
–	20.2 ± 2.3	41.4 ± 3.2	2.05 ± 0.11
Oligomycin	24.1 ± 1.6	50.8 ± 2.0	2.11[a] ± 0.08
Oligomycin + NEM	22.6 ± 2.3	44.1 ± 2.7	1.95 ± 0.20
Oligomycin + Mersalyl	20.7 ± 2.0	39.3 ± 3.5	1.91 ± 0.13
Oligomycin + phosphate	23.5 ± 1.5	51.0 ± 3.8	2.17 ± 0.16
Oligomycin + NEM + phosphate	18.8 ± 1.9	34.6 ± 2.8	1.84[a] ± 0.13
Oligomycin + phosphate (2 min) + NEM	23.6 ± 2.4	52.2 ± 3.3	1.96[a] ± 0.22

For incubation medium see section 2. Other additions: 4 μM oligomycin; 100 μM phosphate; mersalyl 20 nmol/mg protein; NEM 40 nmol/mg protein. NEM was added 2 min before valinomycin or phosphate addition. Phosphate, when not otherwise indicated, was added together with valinomycin. Figures are means ± SD for ⩾ 6 expt ([a]) corrected for H^+ leak

orientation of the phosphate-transport system [17,18]. Only a negligible change in the intramitochondrial concentration of phosphate was found after valinomycin addition, showing that no phosphate transport occurred during the Ca^{2+}-uptake process. In the presence of NEM an immediate but small increase of pH after valinomycin addition was observed.

The addition of valinomycin induced a moderate degree of apparent shrinkage of the mitochondria which was almost the same in the presence or absence of NEM (not shown).

Figure 2 shows the effect of a further addition of calcium on the H^+-fluxes when all the calcium, released as a consequence of addition of antimycin A, was taken up again by valinomycin addition. The pH-increase sharply diminished when calcium was added and the pH began to rise again only when all the calcium added to the medium had been completely taken up by mitochondria.

The rates of K^+-ejection and Ca^{2+}-uptake are shown in table 1. The rates were measured immediately after addition of valinomycin. The ratio of initial rates of K^+-ejected/Ca^{2+} taken up was very close to 2. In the presence of oligomycin a slight increase of the rate of Ca^{2+}-uptake was observed. Phosphate added together with valinomycin did not affect the rate of Ca^{2+}-uptake. When phosphate was added 2 min before NEM and the latter compound 2 min before valinomycin, the rate of Ca^{2+}-uptake was slightly higher. On the contrary when NEM was added before phosphate the rate of valinomycin-induced calcium re-uptake was slower. In all cases the K^+/Ca^{2+} ratio was close to 2.

4. Discussion

The present data demonstrate that the translocation of calcium into non-respiring valinomycin-treated mitochondria proceeds by a process in which 2 positive charges are carried by each Ca^{2+}, confirming earlier less direct experiments [8–10]. These results are in agreement with those obtained with respiring mitochondria [1–7].

A K^+/Ca^{2+} ratio close to 2 is obtained also in the presence of NEM or mersalyl which inhibit the mitochondrial uptake of phosphate (see [19]), indicating that under our experimental conditions no phosphate is necessary for calcium uptake. Also direct measurements of intramitochondrial phosphate concentration showed no co-transport of Ca^{2+} phosphate.

The addition of phosphate to the medium caused a negligible change in the rate of calcium uptake; whereas in the presence of NEM, phosphate added after NEM caused a decrease of the rate of Ca^{2+}-uptake and phosphate added before NEM caused an increase in the rate of Ca^{2+}-uptake. These results indicate that it is the phosphate distribution across the mitochondrial membrane which affects the Ca^{2+}-transport and that phosphate has no direct effect on

the activity of the transport system. Similar conclusions were reached [20] for rat heart mitochondria.

NEM added by itself induced a slight release of Ca^{2+} from mitochondria parallel to an alkalinization of the medium. In the absence of NEM there is no significant pH-change during the K^+/Ca^{2+} exchange, actually an apparent competition between Ca^{2+} and H^+ with regard to their uptake in exchange for K^+ released was observed. Since the experiments were carried out in the presence of oligomycin, the H^+-uptake should occur through 'proton-channels' distinct from that of ATPase. It has been claimed that the effect of NEM on Ca^{2+}-release can hardly be ascribed to a membrane labilization [21]; however, the pH-change observed after addition of antimycin and valinomycin in the presence of NEM suggests that this compound causes a modification of the mitochondrial membrane making it more permeable to the protons.

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