Parallel efflux of Ca^{2+} and P_i in energized rat liver mitochondria

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Addition of Ruthenium Red to energized rat liver mitochondria that have previously accumulated Ca^{2+} and phosphate from the external medium induces a parallel efflux of both these ions. Mersalyl or dithioerythritol, which decrease Ruthenium Red-insensitive Ca^{2+} efflux, also decrease phosphate efflux to the same extent. Conversely diazenedicarboxylic acid bis(*NN*-dimethylamide) (DDBA), which increases the Ruthenium Red-induced Ca^{2+} efflux concurrently increases phosphate release. Dithioerythritol and DDDBA, reducing and oxidizing agents of thiol groups respectively, modify Ca^{2+} and P_i efflux without penetrating the mitochondrial inner membrane. Under all the adopted conditions the membrane potential is preserved. The release of resting respiration and the parallel efflux of Mg^{2+} and adenine nucleotides, events closely correlated to Ca^{2+} cycling, are equally prevented either by mersalyl, which inhibits phosphate transport, or dithioerythritol; DDDBA has the opposite effect. These findings and the observation that phosphate uptake by liver mitochondria is completely prevented by Ruthenium Red suggest that Ca^{2+} and phosphate transport in energized liver mitochondria are closely related and dependent on the redox state of membrane-bound thiol groups.

It is generally accepted that mitochondria possess not only a uniport for Ca^{2+} uptake down its electrochemical gradient, but also an independent pathway for Ca^{2+} release against the electrochemical gradient (for review, see Nicholls & Crompton, 1980). The activity of the efflux pathway in mitochondria from excitable tissues is Na^+ -dependent (Crompton *et al.*, 1978), whereas in liver mitochondria the mechanism is still unresolved.

It has been previously shown that addition of Ruthenium Red to mitochondria that have accumulated Ca^{2+} and P_i from the external medium induces a parallel efflux of Ca^{2+} and P_i . It was then presumed that the efflux of Ca^{2+} from liver mitochondria can occur in co-transport with P_i (N. Siliprandi *et al.*, 1979a). The results reported in the present paper add further evidence to this assumption, showing that both Ca^{2+} and P_i movements in energized mitochondria are simultaneously and equally affected by agents that specifically modify Ca^{2+} or phosphate transport respectively. In particular, it will be shown that mersalyl, a known inhibitor of P_i transport, also inhibits Ca^{2+} cycling and the related Mg^{2+} and adenine nucleotide efflux (Zoccarato *et al.*, 1981). Moreover DTE and

DDDBA, reducing and oxidizing agents respectively of membrane-bound thiol groups (N. Siliprandi *et al.*, 1979b), have opposite effects on both Ca^{2+} and P_i movements and on Mg^{2+} and adenine nucleotide efflux from liver mitochondria.

Experimental

Rat liver mitochondria were isolated in 0.25M-sucrose and 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH 7.4) by the method of Schneider (1956). Mitochondrial protein concentration was assayed by the biuret method (Gornall *et al.*, 1949).

Mitochondrial incubations were carried out at 20°C with 1mg of mitochondrial protein/ml in the following standard medium: 200mM-sucrose, 10mM-Hepes (pH 6.8), 5mM-sodium succinate, 1.25μM-rotenone. Final Ca^{2+} concentration was brought to 30μM after having determined Ca^{2+} content of media by atomic spectroscopy.

Ca^{2+} transport was measured with a Ca^{2+} -selective electrode (Radiometer F2112), with a calomel electrode (Radiometer K401) as the response electrode. The logarithmic response of the Ca^{2+} electrode was linearized by connecting the electrodes to a Beckman Selection TM-5000 Ion Analyzer.

Abbreviations used: DDDBA, diazenedicarboxylic acid bis-(*NN*-dimethylamide) ('diamide'); DTE, dithioerythritol; TPMP, triphenylmethylphosphonium.

Membrane potential ($\Delta\psi$) was determined in parallel experiments by adding $1\mu\text{M}$ - $[^{14}\text{C}]\text{TPMP}$ bromide ($0.016\mu\text{Ci/ml}$) to the standard medium. At defined times 0.8 ml portions of the incubation mixture were withdrawn and layered on to 0.4 ml of silicone oil [AR 100/AR 150 (2:1); Wacker-Chemie G.m.b.H., Munich, Germany] and centrifuged for 60 s in an Eppendorf 3200 centrifuge. The mitochondrial pellet was dissolved in 1 mM-sodium EDTA, 0.1% NaCl, 0.9% deoxycholate (pH 7.4) and then added to Packard Instagel solution. The distribution of the radioactivity between pellet and supernatant was determined in a Beckman LS-100C spectrometer. The concentration of TPMP in the matrix was calculated by assuming a volume of $0.4\mu\text{l/mg}$ of protein.

To determine P_i fluxes, at intervals 1.3 ml samples of the incubation medium were transferred into Eppendorf 3200 centrifuge tubes containing ice-cold *N*-ethylmaleimide (100nmol/mg of protein) and centrifuged (12000rev/min). The pellet was rinsed twice with ice-cold 0.25M -sucrose and extracted with 10% trichloroacetic acid. P_i content was determined by the method of Baginski *et al.* (1967).

O_2 uptake was measured with a Clark oxygen electrode.

Mg^{2+} movements were estimated by atomic-absorption spectroscopy of the supernatant (Siliprandi *et al.*, 1977). Mitochondrial adenine

nucleotide efflux was determined as previously described (Zoccarato *et al.*, 1981).

Results

When rat liver mitochondria were added to a medium containing 1.5mM - P_i and $30\mu\text{M}$ - Ca^{2+} , Ca^{2+} was rapidly accumulated and slowly released on addition of Ruthenium Red (Fig. 1a). Addition of mersalyl, after Ca^{2+} and P_i have been accumulated, inhibited Ca^{2+} efflux. The presence in the medium of DTE induced a considerable decrease in Ca^{2+} efflux, which, on the contrary, was enhanced by DDBA. It is noteworthy that in the absence of Ruthenium Red, mitochondria were able to retain accumulated Ca^{2+} (Fig. 1a, broken line) also in the presence of each of the three agents (very low concentrations of DDBA were used for this purpose). This implies that membrane potential remained sufficiently high to preclude the spontaneous efflux of accumulated Ca^{2+} from the matrix space. Measurements of $\Delta\psi$ with TPMP in parallel experiments have confirmed that membrane potential was not substantially affected by any of the imposed conditions. This observation is in full agreement with the finding of Nicholls & Brand (1980) that the retention of Ca^{2+} correlates closely with the maintenance of membrane potential. Hence it is very unlikely that the observed Ruthenium Red-dependent Ca^{2+} efflux

Fig. 1. Parallel efflux of Ca^{2+} (a) and P_i (b) from rat liver mitochondria induced by Ruthenium Red in the presence of external phosphate

Rat liver mitochondria (1mg/ml) were suspended in the standard medium containing 1.5mM -sodium phosphate (pH 6.8). Temperature was 20°C . When present concentrations were: DTE, 1mM ; DDBA, $25\mu\text{M}$; Ruthenium Red (RR), $0.16\mu\text{M}$. When indicated, $0.16\mu\text{M}$ -Ruthenium Red was added at 5 min and $10\mu\text{M}$ -mersalyl (MERS) at 3 min. $\Delta\psi$ measurements, Ca^{2+} and P_i fluxes were determined in parallel. Values in parentheses are $\Delta\psi$ (mV) determined at 4.5 min and 10 min. P_i data are mean values of measurements made in triplicate.

promoted by P_i reflects unspecific mitochondrial membrane damage.

Ruthenium Red also induced an efflux of P_i simultaneous and comparable with that of Ca^{2+} (Fig. 1b). Analogously, mersalyl strongly decreased P_i efflux induced by Ruthenium Red. Conversely the uptake of P_i was prevented by Ruthenium Red (Fig. 1b, broken line). Therefore Ca^{2+} and P_i transport appear to be complementary in the sense that the inhibition of Ca^{2+} transport by Ruthenium Red prevents P_i influx, and the inhibition of P_i transport by mersalyl in turn inhibits Ca^{2+} release. DTE and DDBA exert an opposite effect also on P_i efflux induced by Ruthenium Red (Fig. 1b). Since neither DTE, nor DDBA, modify the transport of Ca^{2+} and P_i into mitochondria, necessarily these two reagents must affect specifically the efflux pathway of the two ions.

In the absence of added P_i the Ruthenium Red-insensitive Ca^{2+} efflux was very low and insensitive to mersalyl, DTE and DDBA (Fig. 2a). It would then appear that the action of these agents is dependent on the external P_i . Under the same conditions (Fig. 2b) the efflux of endogenous P_i was negligible.

Taken together these results give further and more consistent evidence in support of our previous assumption that Ca^{2+} and P_i efflux from energized mitochondria are closely related (N. Siliprandi *et al.*, 1979a).

DTE and mersalyl, on the one hand, and DDBA, on the other, also exhibited opposite effects on the release of resting respiration promoted by P_i (Fig. 3). Such a release is due only in part to the energy dissipation caused by the increased rate of Ca^{2+} uptake compensating for the increased efflux induced by added P_i . In fact a Ca^{2+} recycling rate much higher than can be deduced from the

Ruthenium Red-insensitive efflux rate (Fig. 1) would be needed to support the observed respiration increment. However, it is certain that Ca^{2+} cycling is the cause of the increased State-4 respiration, since

Fig. 3. Release of resting respiration of rat liver mitochondria induced by phosphate

Experimental conditions were as in Fig. 1; final volume was 2 ml. For definition of abbreviations, see the legend to Fig. 1. The broken line refers to experiments in the presence of Ruthenium Red.

Fig. 2. Efflux of Ca^{2+} and P_i from rat liver mitochondria induced by Ruthenium Red in the absence of external phosphate. Experimental conditions were as in Fig. 1, except that phosphate was absent. For definition of abbreviations see the legend to Fig. 1.

Fig. 4. Efflux of Mg^{2+} (a) and adenine nucleotides (b) from rat liver mitochondria induced by external phosphate. Experimental conditions were as in Fig. 1. When present, $10 \mu M$ -methylsalicyl was added to the medium before addition of rat liver mitochondria. For definition of abbreviations, see the legend to Fig. 1.

addition of Ruthenium Red completely abolished the stimulation (Fig. 3; see also N. Siliprandi *et al.*, 1979a; D. Siliprandi *et al.*, 1977, 1978).

This is also true for the even stronger stimulation of resting respiration induced by DDBA (Fig. 3).

It has been previously shown that the presence of Ca^{2+} and P_i induces a parallel efflux of endogenous Mg^{2+} and adenine nucleotides from rat liver mitochondria closely related to Ca^{2+} cycling (Zoccarato *et al.*, 1981). Indeed the results shown in Fig. 4 indicate that the simultaneous efflux of Mg^{2+} and adenine nucleotides from liver mitochondria promoted by external Ca^{2+} and P_i was stimulated by DDBA and prevented either by DTE or mersalyl. Since both Mg^{2+} and adenine nucleotide efflux and the release of resting respiration reflect Ca^{2+} cycling, these results provide indirect evidence that such a cycling can be inhibited by blocking Ca^{2+} or P_i transport with Ruthenium Red and mersalyl respectively, or by modifying in the opposite way the permeability properties of the mitochondrial membrane by DTE and DDBA respectively.

It was previously reported that when approx. 60% of endogenous Mg^{2+} was lost, mitochondria became uncoupled (D. Siliprandi *et al.*, 1979). Such a transition state explains the progressive acceleration of resting respiration induced by DDBA (Fig. 3), which, in the absence of Ruthenium Red, induces a faster Mg^{2+} release from mitochondria (Fig. 4).

It is noteworthy that, unlike P_i , acetate failed to evoke any of the above events.

Discussion

The results reported here show that external Ca^{2+} and P_i , at concentrations not far from those existing

in the cytosol, promote in rat liver mitochondria a series of correlated events that are dependent on Ca^{2+} movements.

The requirement of P_i transport in evoking these events (Ruthenium Red-insensitive efflux of accumulated Ca^{2+} and P_i , release of resting respiration, efflux of endogenous Mg^{2+} and adenine nucleotides) results from the observation that mersalyl abolishes all the described effects (see also Roos *et al.*, 1980). This is contrary to the observation by Harris *et al.* (1979) that mersalyl increases the Ruthenium Red-insensitive Ca^{2+} efflux from heart mitochondria. This discrepancy is very probably due to the different concentrations of the agent used. Indeed we have also observed that when mersalyl was added to mitochondria in excess of the amount strictly needed to prevent P_i transport, Ca^{2+} efflux was increased, owing to an uncoupling effect.

As shown by the preservation of $\Delta\psi$ and the capability of mitochondria to retain accumulated Ca^{2+} , the combined action of P_i and Ca^{2+} , described above occurs without appreciable change in membrane potential. Therefore it can be assumed (Nicholls & Crompton, 1980) that Ca^{2+} efflux observed in our conditions is part of the physiological efflux pathway.

The most important consideration emerging from the reported results concerns the close and constant parallelism between Ca^{2+} and P_i efflux from energized mitochondria (cf. *a* and *b* in Fig. 1).

The Ca^{2+}/P_i stoichiometry of about 0.6, as can be deduced from the data of Fig. 1, is not incompatible with the assumption of a Ca^{2+}/P_i co-efflux. The amount of P_i released in excess of that of Ca^{2+} is partly accounted for by a concurrent efflux of mitochondrial Mg^{2+} and K^+ (N. Siliprandi *et al.*,

1978; Zoccarato *et al.*, 1981). The assumption of Ca^{2+} and P_i co-efflux is further strengthened by the observation that the $\text{Ca}^{2+}/\text{P}_i$ ratio is maintained closely constant under the different experimental conditions of either increasing or decreasing the efflux rate of the two ions (Fig. 1).

The parallel efflux of Ca^{2+} and P_i is also in agreement with the results shown in Fig. 6(b) of Roos *et al.* (1980), wherein Ruthenium Red-insensitive Ca^{2+} efflux occurs simultaneously with P_i efflux. The conclusion of these latter authors that the release of phosphate precedes that of Ca^{2+} is drawn from experiments carried out in the absence of Ruthenium Red.

The close parallelism between Ca^{2+} and P_i flux in liver mitochondria also emerges from the observation that DTE inhibits and DDBA stimulates the Ruthenium Red-insensitive efflux of both these ions. It is important to outline that both DDBA (used here at low concentration) and DTE are unable to penetrate the mitochondrial inner membrane and do not affect the redox state of mitochondrial nicotinamide nucleotide pool (N. Siliprandi *et al.*, 1979b). Therefore this action on Ca^{2+} and P_i efflux is the

result of opposite changes in the redox state of membrane-bound thiol groups.

That P_i facilitates Ca^{2+} uptake and increases the capacity of mitochondria to accumulate Ca^{2+} (Rossi & Lehninger, 1963) has been known for some time. However, as pointed out by Fiskum & Lehninger (1980), P_i transport is only indirectly coupled to Ca^{2+} uptake. Conversely, as indicated by the present results, P_i uptake is directly coupled to Ca^{2+} transport, since Ruthenium Red, the specific inhibitor of Ca^{2+} uptake, also prevents P_i transport into mitochondria (Fig. 1b). Thus it would appear that P_i transport in mitochondria is driven by Ca^{2+} movements.

The proposed co-transport of Ca^{2+} and P_i does not exclude other 'efflux mechanisms' such as $\text{H}^+/\text{Ca}^{2+}$ exchange. Ca^{2+} efflux occurring in the absence of added P_i (Fig. 2a) may be accounted for by this $\text{H}^+/\text{Ca}^{2+}$ exchange.

However, the concurrent efflux of Ca^{2+} and P_i seems to be predominant, if not exclusive, when P_i is present in the external medium, and particularly when the pH of the medium is low, as in our conditions (pH 6.8).

In conclusion, we assume that Ca^{2+} efflux from energized mitochondria occurs in co-transport with P_i in an electroneutral process, compatible with the preservation of the transmembrane potential. It can also be postulated that Ca^{2+} and P_i are concurrently transported into and out of mitochondria in a cycle, whereby P_i enters in a protonated form and flows out associated with Ca^{2+} (Fig. 5). Such a correlated flux of the two ions is inhibited by Mg^{2+} (N. Siliprandi *et al.*, 1979a) and modulated by the redox state of membrane-bound thiol groups.

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Fig. 5. A model for mitochondrial cyclic transport of Ca^{2+} and P_i .

Ca^{2+} and P_i enter on their own carriers sensitive to Ruthenium Red (RR) and mersalyl (MERS) respectively. The parallel efflux of Ca^{2+} and P_i out of mitochondria is insensitive to RR and sensitive to mersalyl. Ca^{2+} and P_i released are re-accumulated by energized mitochondria. Reducing and oxidizing agents respectively inhibit and stimulate Ca^{2+} and P_i efflux. Abbreviations used: RED, reducing agent (DTE); OX, oxidizing agent (DDBA).

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