RESPIRATORY CONTROL IN SUBMITOCHONDRIAL PARTICLES AND Ca^{++} TRANSPORT

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Received October 13, 1967

It has been repeatedly stated that submitochondrial particles prepared by sonic disruption of mitochondria show no effects of Ca^{++} similar to those observed in intact mitochondria. Neither stimulation of respiration nor uncoupling by Ca^{++}, nor accumulation of Ca^{++} has been observed (Vasington, 1963; Brierley et al., 1964; Chance, 1965). An explanation of these findings has been based on the observation that morphologically these particles appear "inside-out" (Lee and Ernster, 1966; Mitchell, 1966). Unidirectional transport as predicted by the concepts of the chemiosmotic hypothesis would demand that ion flow in such inverted submitochondrial particles proceed from the "inside" to the medium (Mitchell, 1966).

Submitochondrial particles obtained from light layer mitochondria of beef heart (Green et al., 1957) by sonication in the presence of pyrophosphate buffer (Racker, 1963) showed marked stimulation of DPNH oxidation by the addition of Ca^{++} (Figure 1). Uncoupling agents such as CCP[1] or 2,4-dinitrophenol had comparatively little effect either in the presence or absence of Ca^{++}. There was little or no stimulation of the rate of succinate oxidation by either CCP or Ca^{++}.

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*This work was supported by Public Health Research Grant No. CA-03463-08 from the National Cancer Institute.

[1]Abbreviations: CCP for carbonylcyanide p-trifluoromethoxyphenylhydrazone; SMP for submitochondrial particles.

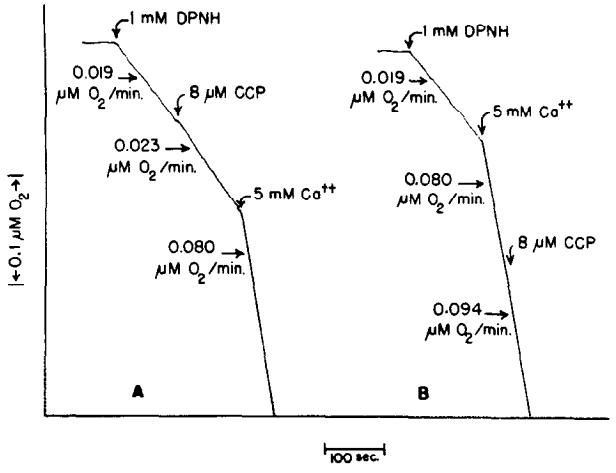

Figure 1. Effects of Ca^{++} and CCP on Respiration in Submitochondrial Particles.

The system contained 380 μg of submitochondrial protein, 25 mM Tricine, pH 7.4, and 0.25 M sucrose in a final volume of 1.1 ml. The temperature was 21°. Additions of DPNH (1 mM), CCP (8 μM) and Ca^{++} (5 mM) and corresponding values for oxygen uptake are indicated alongside the tracings.

The possibility that only DPNH oxidation could be used for energy production in these particles was eliminated by measuring oxidative phosphorylation. As shown in Table I, DPNH and succinate were oxidized with P:O ratios of 1.05 and 0.60 respectively. In the presence of 3 mM Ca^{++} the P:O ratio with DPNH fell to 0.57, whereas that with succinate remained essentially unchanged. CCP completely uncoupled phosphorylation with either substrate in the absence or presence of Ca^{++}. Dicumarol or 2,4-dinitrophenol also uncoupled oxidative phosphorylation in these particles.

The possibility was then considered that in these submitochondrial particles the uncoupling action of Ca^{++} might be associated with an active ion transport similar to that in intact mitochondria. It was found that these particles indeed accumulated large amounts of Ca^{++} under appropriate

TABLE I

EFFECTS OF Ca^{++} AND CCP ON OXIDATIVE PHOSPHORYLATION

Components	Additions	O_2 Uptake in 10 min μatoms	P_i Uptake in 10 min μmoles	P:O
1. DPNH Oxidation				
a) SMP	none	0.248	0.171	0.69
b) SMP + F_1	none	0.284	0.299	1.05
c) SMP + F_1	Ca^{++}, 3 mM	0.586	0.334	0.57
d) SMP + F_1	CCP, 8 μM	0.367	0.000	0.00
2. Succinate Oxidation				
a) SMP	none	0.558	0.129	0.23
b) SMP + F_1	none	0.352	0.211	0.60
c) SMP + F_1	Ca^{++}, 3 mM	0.378	0.237	0.63
d) SMP + F_1	CCP, 8 μM	0.396	0.000	0.00

In a final volume of 0.03 ml, a mixture containing 500 μg of light-layer submitochondrial particles and 0.25 M sucrose, 1.32 mM EDTA, 2.64 mM ATP and 13.2 mM Tricine, pH 7.4, either with or without 50 μg of F_1 (as indicated) were incubated for 20 min at 21°. After another 20 min incubation at 2°, 0.015 ml of the mixture was then placed into a polarograph chamber in a final volume of 1.1 ml containing 2.2 μmoles of $MgSO_4$, 0.55 μmole of EDTA, 5.5 μmoles of Tricine, pH 7.4, 35.2 μmoles of glucose, 1.1 μmoles of ATP, 16.5 units of dialysed hexokinase, 2.2 mg of dialysed bovine serum albumin and 15 μmoles of purified $^{32}P_i$ (Schatz and Racker, 1966), pH 7.4 (6.3×10^4 cpm per μmole). The temperature was 21°. Additions were made as noted in the table and after equilibration, respiration was initiated by addition of substrate. DPNH was generated by a system containing 10 μmoles of ethanol, 18 μg of alcohol dehydrogenase, 110 μg of yeast aldehyde dehydrogenase, 0.5 μmoles of dithiothreitol and 0.6 μmole of DPN which was added last (unpublished method). Similar results were obtained with DPNH (1 mM) as substrate, but with somewhat lower P:O ratios. When succinate was used as substrate, the final concentration was 15 mM.

conditions. It can be seen from Table II that during DPNH oxidation these particles can accumulate about 1 μmole of Ca^{++} per mg protein in 20 minutes. (However, most of the accumulation takes place within the first 5 to 10 minutes.) Omission of DPNH served as a control with values

TABLE II

Ca^{++} ACCUMULATION IN SUBMITOCHONDRIAL PARTICLES

Substrate	Inhibitor	Ca^{++} Accumulation (mμmoles/mg protein/20 min)
Experiment 1		
None	none	25
DPNH, 15 mM	none	950
DPNH, 15 mM	CCP (50 μM)	930
DPNH, 15 mM	Dinitrophenol (20 μM)	900
DPNH, 15 mM	Rotenone (10 μM)	155
Experiment 2		
None	none	130
Succinate, 100 mM	none	102
Succinate, 100 mM + ATP, 10 mM	none	130

The reaction mixture contained 0.25 M sucrose, 20 mM $MgSO_4$, 7 mM KPO_4, pH 7.2, and beef heart light layer submitochondrial particles (3 mg/ml). After 5 min at 21°, 5 μmoles per ml of $^{45}CaCl_2$ (8×10^4 cpm/ml) were then added and after addition of substrate the mixture was shaken vigorously for 20 min. The reaction was stopped by rapid filtration of 1 ml sample on HA 0.45 μ Millipore filters. Aliquots (50 μl) of the incubation mixture and of the filtrate were then plated on planchets, dried, and counted in a Nuclear-Chicago gas-flow counter. Ca^{++} accumulation was calculated from the difference in cpm before and after filtration.

well below 200 mμmoles of bound Ca^{++} per mg protein. Similar "non-specific" binding of Ca^{++} has been observed by other investigators (Gear et al., 1967). Addition of rotenone (10 μM) or antimycin A (7 μM) strongly inhibited DPNH oxidation and Ca^{++} accumulation. In contrast to DPNH oxidation, succinate oxidation did not result in Ca^{++} accumulation significantly above the control values either in the presence or absence of ATP. Ca^{++} accumulation in submitochondrial particles was not inhibited by CCP in concentrations which completely uncoupled oxidative phosphorylation. Dicumarol (10 to 200 μM) or 2,4-dinitrophenol (20 to

200 μM) also had no effect on Ca^{++} accumulation. Since these results were unexpected the experiments were carried out under a variety of conditions with respect to time, concentration of uncouplers and prior exposure of the particles to the uncoupler with essentially the same results.

In the experiments shown in Table I, F_1 was added to the particles since it increased the P:O ratio, but it had no effect on Ca^{++} accumulation. It should also be mentioned that parallel experiments were carried out with similar submitochondrial particles from heavy layer mitochondria. In each case the results were qualitatively the same. Data with submitochondrial particles from light layer are presented here primarily because the stimulation of respiration by Ca^{++} was more pronounced than in heavy layer submitochondrial particles.

The data presented immediately raise questions with respect to the site of action of Ca^{++} and uncouplers such as CCP. A current formulation of the chemical hypothesis is shown below with A and B representing members of the respiratory chain:

$$AH_2 + B + X \rightleftharpoons A\sim X + BH_2$$

$$A\sim X + Y \rightleftharpoons X\sim Y + A$$

$$X\sim Y + P_i + ADP \rightleftharpoons X + Y + ATP$$

Uncouplers such as CCP or dinitrophenol are visualized to act at a site close to the respiratory chain ($A\sim X$), while Ca^{++} is postulated to interact with $X\sim Y$ (Chance, 1965; Lee and Ernster, 1966). The above described experiments suggest, however, that the sites of action may be reversed, with Ca^{++} acting at the $A\sim X$ site, CCP acting on $X\sim Y$, and the conversion of $A\sim X$ to $X\sim Y$ being rate-limiting.

The data further indicate that Ca^{++} uncouples phosphorylation specifically associated with DPNH oxidation, perhaps only at the first

site. The same site appears to be operative in the transport of Ca^{++} in these particles.

In intact mitochondria succinate oxidation supports Ca^{++} accumulation (Brierley et al., 1964; Chance, 1965; Engström and DeLuca, 1964; Vasington and Murphy, 1962; Drahota et al., 1965; Hittelman et al., 1967). It remains to be examined whether submitochondrial particles have lost this capacity, or whether in mitochondria an energy driven reversal at the first site is responsible for Ca^{++} accumulation. A requirement for adenine nucleotides for succinate-driven Ca^{++} accumulation (Vasington and Murphy, 1962; Hittelman et al., 1967) would favor the latter possibility, and an exploration of this question is now in progress.

In view of the findings presented here it is necessary to reexamine the formulations of both the chemical and chemiosmotic hypotheses. The former would need revision with respect to site specificity, since it seems that $X \sim Y$, the presumed common intermediate, is not a likely candidate for interaction with Ca^{++}. It is even more difficult to reconcile these findings with the chemiosmotic hypothesis. In this connection it should be emphasized that only minor stimulatory effects were observed with NaCl or KCl at ionic strengths that in the case of $CaCl_2$ gave 4 to 5 fold stimulation.

SUMMARY

Submitochondrial particles obtained from beef heart mitochondria by sonic disruption accumulated Ca^{++} during DPNH oxidation but not during succinate oxidation. Rotenone or antimycin inhibited the accumulation of Ca^{++}. Oxidation of DPNH but not of succinate was stimulated 4 to 5 fold by Ca^{++}. Uncouplers such as carbonylcyanide phenylhydrazone did not stimulate the oxidation of either DPNH or succinate and did not prevent the accumulation of Ca^{++}. These findings could not be explained by a lack of energy production with succinate

as substrate, since both DPNH and succinate catalyzed oxidative phosphorylation with P:O ratios of 1.0 and 0.6 respectively.

ACKNOWLEDGEMENTS

We wish to acknowledge the competent technical assistance of Mrs. J. Saltzgaber. We also thank Dr. W. B. Jacoby for information with regard to the preparation of yeast aldehyde dehydrogenase.

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