EFFECT OF Ca^{2+} ON COUPLING OF RAT LIVER MITOCHONDRIA

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1. Introduction

Mitochondrial Ca^{2+} transport is an energy requiring process that gives rise to stimulation of oxygen consumption. Whether the respiratory rate returns to the basal rate after Ca^{2+} uptake by the mitochondria is completed depends on the Ca^{2+}/protein ratio as well as on the composition of the medium [1–3]. However, as it will be shown in this paper, the rate of respiration after Ca^{2+} application is also affected by the order of the addition of Ca^{2+} and the respective substrate. The results demonstrate that succinoxidase activity after accumulation of small amounts of Ca^{2+} depends on the functional state of the mitochondria at the moment of Ca^{2+}-addition.

2. Methods

Liver mitochondria from Wistar-rats were prepared according to Schneider [4]. The rate of oxygen consumption was determined by a vibrating Pt-electrode in a closed system. Swelling of mitochondria was measured with a recording spectrophotometer at 520 nm. $^{45}Ca^{2+}$-uptake experiments were performed in small centrifuge tubes. After incubation the mitochondria were spun down rapidly in a microcentrifuge and Ca^{2+}remaining in the supernatant was measured by liquid scintillation counting. Protein was determined according to the method of GORNALL and associates [5].

Abbreviations: EGTA = ethylene glycol bis- (β-aminoethyl-ether)-*N*, *N'* tetraacetic acid.

3. Results

In a sucrose-KCl-medium containing not more than 5 mM inorganic phosphate, mitochondria return to state 4 after accumulation of at most 200 nmoles Ca^{2+}/mg protein. This behaviour is only observed only in those cases where Ca^{2+} is added after rotenone and succinate or after rotenone alone. In the latter case Ca^{2+} is followed by succinate as is shown in fig. 1 (upper curve). However, when Ca^{2+} is applied to the mitochondrial suspension prior to rotenone and succinate, whether the mitochondria are able to return to the basal respiration or not (lower curves in fig. 1) depends on the period of incubation with Ca^{2+} in absence of substrate and rotenone. Succinate oxidation appears to be uncoupled when rotenone and succinate are added 60 sec after Ca^{2+} or later.

To measure the uncoupling effect of Ca^{2+}, ADP was added one minute after succinate in another set of experiments similar to that in fig. 1 (not represented in the graph). About 200 nmoles of Ca^{2+}/mg protein have been found to be necessary for a complete release of respiratory control, when Ca^{2+} was added after rotenone, whereas 40 nmoles Ca^{2+}/mg protein are required when Ca^{2+} is added prior to rotenone and succinate.

The results of experiments with $^{45}Ca^{2+}$ are presented in fig. 2. In the absence of rotenone and succinate 40% of $^{45}Ca^{2+}$ is taken up immediately (upper curve). After a short accumulation phase, however, Ca^{2+} is again partially released. Addition of rotenone and succinate is followed by a small transient uptake only. In the control experiment (lower curve) only 25% of the Ca^{2+} is slowly bound to the mitochondria. The

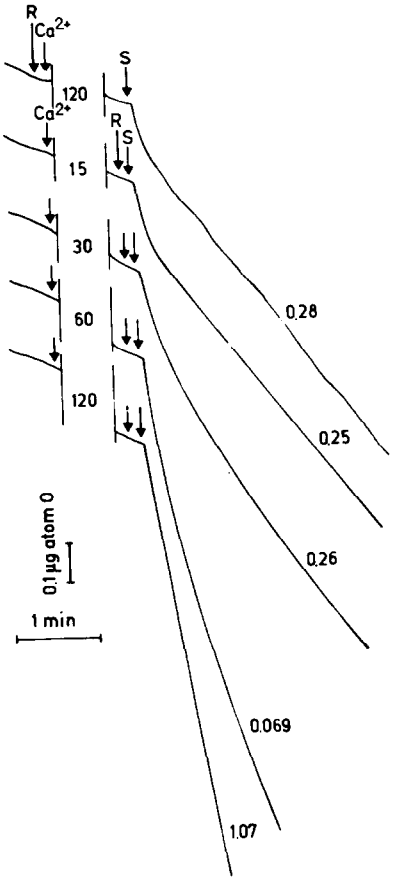

Fig. 1. Succinate oxidation after preincubation of mitochondria with Ca^{2+} in absence and presence of rotenone. Medium: sucrose 100 mM, KCl 75 mM, Tris–HCl (pH 7.5) 20 mM, phosphate 5 mM, $MgCl_2$ 2,5 mM. 2.4 mg protein/ml, total volume 3.3 ml. 25°C. Additions as indicated by the arrows: R = 1.5 µg rotenone/ml, S = 10 mM succinate, Ca^{2+} = 136 µM $CaCl_2$. Numbers in the break indicate the time in seconds between addition of Ca^{2+} and the subsequent addition. The first addition was made 1 min after starting with mitochondria. Numbers at the curves indicate µg atoms O/min.

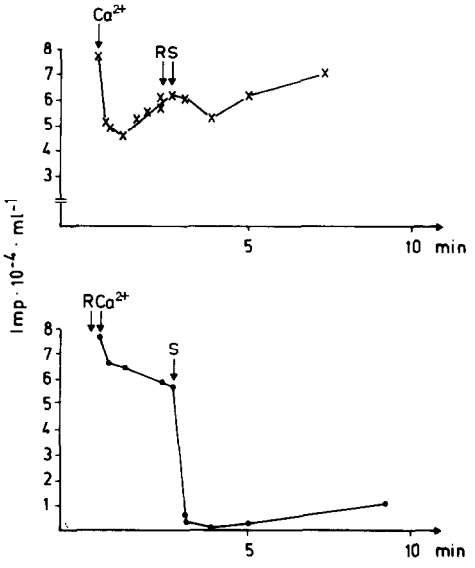

Fig. 2. $^{45}Ca^{2+}$-uptake by mitochondria. Medium see fig. 1. 3.4 mg protein/ml. Ca^{2+} = 120 µM. Other conditions as in fig. 1. See also text.

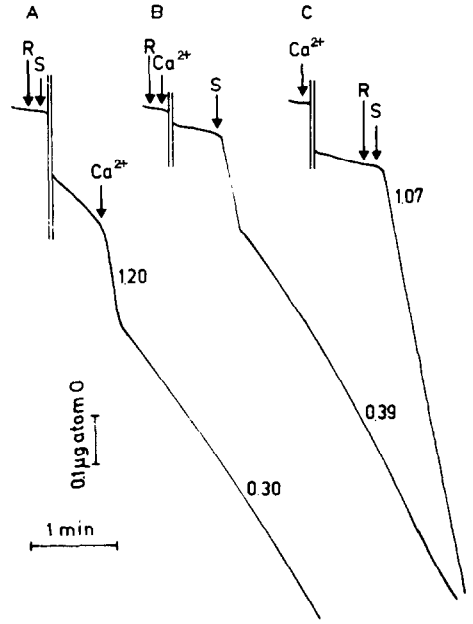

Fig. 3. Effect of bovine serum albumin on Ca^{2+}-induced uncoupling. Medium see fig. 1. Additions: 0.6% bovine serum albumin, 1.7 mg protein/ml. Ca^{2+} = 150 µM. Other conditions as in fig. 1.

rest is completely accumulated after addition of succinate.

From the results of fig. 1 it may be supposed that the activation of mitochondrial phospholipase by Ca^{2+} might be the reason for the uncoupling effect. The results of experiments in presence of bovine serum albumin (fig. 3), however, exclude the possibility that free fatty acids liberated from phospholipids might cause the uncoupling effect. Scherphof and his asso-

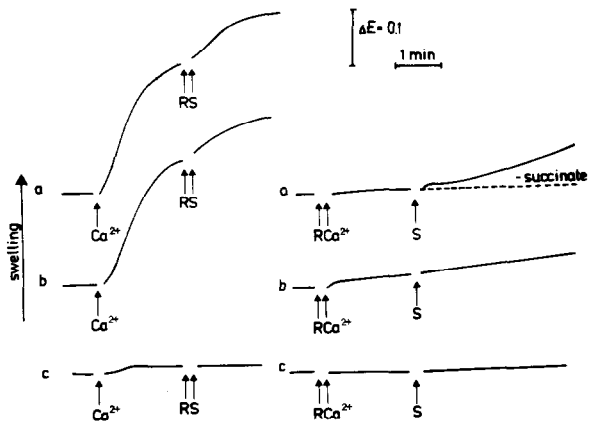

Fig. 4. Effect of chlorpromazine or ruthenium red on Ca^{2+}-induced swelling of mitochondria in the presence and absence of rotenone. Medium see fig. 1. 4.5 mg protein/ml. Ca^{2+} = 260 μM. Further additions to the medium: expt. b: 120 μM chlorpromazine; expt. c: 5.0 nmoles ruthenium red/mg protein. Other conditions as in fig. 1.

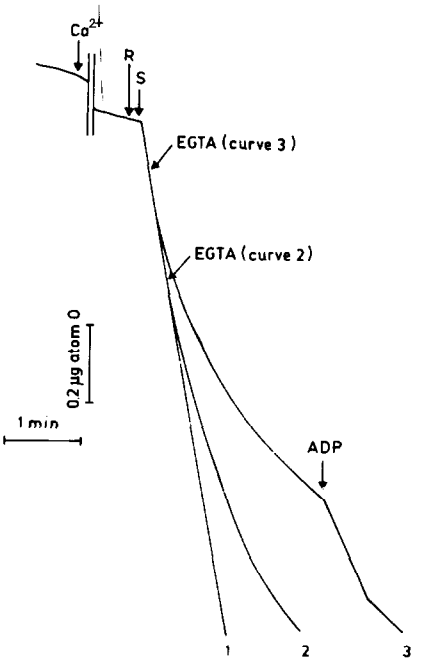

Fig. 5. Influence of EGTA on Ca^{2+}-induced uncoupling. Medium see fig. 1. 2.6 mg protein/ml. Ca^{2+} = 150 μM. EGTA = 1 mM, ADP = 0.14 μM. Other conditions as in fig. 1.

ciates [6] suggested that rather the formation and accumulation of lysophosphatides are apparently responsible for the deleterious effect of phospholipase action on membranes.

Owing to the existence of a close relationship between mitochondrial swelling and extent of hydrolysis of mitochondrial phospholipids [6] in the following experiments the effect of Ca^{2+} on mitochondrial swelling was studied. The results are shown in fig. 4. Ca^{2+} in the absence of rotenone and succinate induces a rapid and significant swelling (experiment a, left hand side), whereas in the control only a slight swelling is observed (right hand side). The dotted line corresponds to an experiment without succinate. For getting further insight whether a phospholipase is involved in the swelling process, the effect of chlorpromazine, which is known as a potent inhibitor of Ca^{2+}-stimulated phospholipases [7,8] was studied (experiment b). Chlorpromazine does not influence the swelling process induced by Ca^{2+} in the absence of rotenone and succinate. It proved to be ineffective up to a concentration of 250 μM. With the local anaesthetic tetracain, which is also an inhibitor of Ca^{2+}-stimulated phospholipases [6], no inhibition of Ca^{2+}-induced swelling could be obtained (results not shown). The specific inhibitor of Ca^{2+}-transport ruthenium red prevents the swelling process induced by Ca^{2+} (experiment c).

In order to evaluate whether the uncoupling effect of Ca^{2+} added prior to rotenone or succinate is irreversible or not, EGTA was added shortly after the addition of succinate (fig. 5). It was found that respiration slowly returns to a lower rate and that respiratory control by ADP is regained. These results provide evidence for the reversibility of the Ca^{2+}-induced changes.

4. Discussion

The results presented in this paper clearly show that uncoupling occurs, when mitochondria have been preincubated with Ca^{2+} before addition of rotenone and succinate, whereas respiration returns to state 4 after accumulation of Ca^{2+}, when rotenone was added prior to Ca^{2+}. Recently, Cittadini and associates have reported a similar result at the cellular level [9]. They

observed an irreversible stimulation of respiration of ascites tumour cells, when Ca^{2+} was accumulated on account of endogenous substrate. In the presence of rotenone and succinate, however, respiration returns to the basal rate after Ca^{2+} uptake has been finished.

Owing to the fact that short preincubation is necessary to render the mitochondria uncoupled the formation of lysophosphatides might be responsible for the Ca^{2+} effects. The involvement of extramitochondrial phospholipases originating from contaminating lysosymes, microsomes or plasma membranes, however, can evidently be ruled out by the following reasons:

1. There is neither uncoupling nor significant swelling in the controls in spite of the higher extramitochondrial Ca^{2+}-concentrations during incubation with rotenone and Ca^{2+}.
2. According to the experiments with ruthenium red, the accumulation of Ca^{2+} appears to be a necessary prerequisite to the observed effect.
3. Lysosomal and microsomal phospholipases are inhibited by Ca^{2+} [10,11], whereas acid lipase is activated only at higher concentrations of Ca^{2+} [12].
4. The effect of extramitochondrial Ca^{2+}-stimulated phospholipases is more pronounced under conditions of inhibited electron transport [7]. In contrast, no swelling could be observed by Ca^{2+} with rotenone – inhibited mitochondria in the absence of succinate (dotted line in experiment a of fig. 4).
5. Extramitochondrial phospholipases sensitive to Ca^{2+} are inhibited by local anaesthetics [6–8]. The rapid swelling induced by Ca^{2+}, however, was not inhibited by chlorpromazine or tetracain.

The possibility remains, therefore, that formation of lysophospholipids by stimulation of the mitochondrial phospholipase A itself might induce the uncoupling effect and swelling process. The lack of inhibition of Ca^{2+}-induced swelling by chlorpromazine might argue against the action of mitochondrial phospholipases. However, phenothiazines at chlorpromazine: protein ratios as they have been used in this study accumulate to a great extent in the boundary phase of membranes [13] so that the possibility remains that they are not able to reach the site of phospholipase action.

The results with ruthenium red indicate that Ca^{2+} accumulation is necessary to induce uncoupling and swelling of mitochondria. Hence, it may be supposed that Ca^{2+}, added in the absence of rotenone and succinate, is accumulated on account of endogenous substrates thus leading to deenergization of mitochondria. This might give rise to leakage of Ca^{2+}, which can not be compensated by an increased activity of the Ca^{2+} pump. This transport cycle might evidently be interrupted by EGTA (fig. 5). As there is no comparable effect of Ca^{2+} accumulated under conditions of the controls, the response of mitochondria towards Ca^{2+} depends obviously on their functional state. This point must be taken into consideration independently of the mechanism by which uncoupling and swelling are induced.

Acknowledgments

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