ACTA CHEMICA SCANDINAVICA 24 (1970) 3697–3700

Inhibition of Phospholipase A-Induced Swelling of Mitochondria in the Energized State

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Energizing rat liver mitochondria by succinate respiration or by addition of adenosine triphosphate inhibited the swelling, induced by added snake venom phospholipase A. Swelling was obtained upon deenergisation by inhibiting respiration or ATP utilisation, respectively. Uncoupling agents stimulate the action of phospholipase A only in energized mitochondria. Swelling could also be prevented by addition of a chelator of calcium ions. Swelling could then be obtained by addition of a slight excess, less than 1 μM, of calcium ions. Energisation did not inhibit the swelling when lanthanum ions were present to inhibit the energy-dependent accumulation of calcium ions by mitochondria. It is concluded that the external concentration of calcium ions, rather than the conformational state of the membrane, determines the response of mitochondria towards added phospholipase A. Deenergized mitochondria release sufficient amounts of their endogenous calcium to activate the enzyme, while energized mitochondria lower the external calcium concentration below that required for activity.

Added snake venom phospholipase A (phosphatide acylhydrolase, EC 3.1.1.4) induces an extensive swelling of mitochondria after a short lag.[1–3] The lag is shortened by anionic uncoupling agents, indicating an increased susceptibility to the lysis induced by this enzyme activity.[3] Weinbach *et al.*[3–5] interpret this and related data as evidence of a conformational change in the membrane, caused by the uncoupling agents. The aim of this study was to investigate the effect of the energy state of the mitochondria on their susceptibility towards added phospholipase A, taking into account the possibility that these responses might be controlled by the concentration of calcium ions in the external medium.

MATERIALS AND METHODS

Rat liver mitochondria were prepared and methods used as described elsewhere.[6] Incubation of mitochondria with phospholipase A was carried out essentially as by Weinbach and Garbus.[3] Phospholipase A was obtained from Koch-Light Laboratories

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Ltd., Colnbrook, England, as *Crotalus adamanteus* venom or as a purified preparation from *Vipera russellii* venom. Both preparations gave the same results.

RESULTS AND DISCUSSION

When endogenous respiration is blocked, as with antimycin, the lag preceding phospholipase A-induced swelling is shortened, (Fig. 1). In these conditions, addition of uncoupling agents has no significant effect upon the lag. It is thus probable that under the experimental conditions of Weinbach and Garbus,[3] the mitochondria were not in a deenergized state before the addition of uncoupling agents.

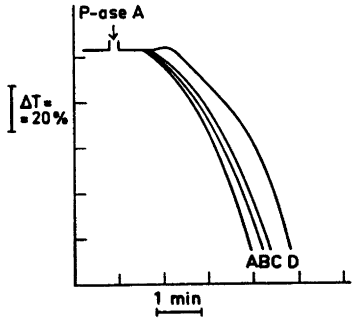

Fig. 1. Effect of dinitrophenol on the phospholipase A-induced swelling of mitochondria in the presence and absence of antimycin. 20 μl of a 0.2 % solution of *Crotalus adamanteus* venom phospholipase (P-ase A) was added to a mitochondrial suspension (protein 0.7 mg/ml) in 225 mM mannitol, 75 mM sucrose, 10 mM Tris, pH 7.9, final volume 3.0 ml. A. In the presence of 67 μM dinitrophenol (DNP) and 1.7 μg/ml antimycin. B. DNP omitted. C. Antimycin omitted. D. Control without DNP and antimycin.

Energizing the mitochondria by succinate respiration postponed or prevented the swelling induced by phospholipase A (Fig. 2). Blocking respira-

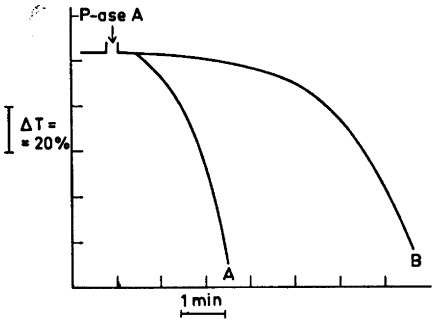

Fig. 2. Effect of succinate respiration on the phospholipase A-induced swelling of mitochondria. Experimental conditions as in Fig. 1. A. Succinate omitted or in the presence of succinate plus cyanide or carbonyl cyanide *p*-trifluoromethoxy phenylnydrazone (FCCP). B. In the presence of 6.7 mM succinate alone.

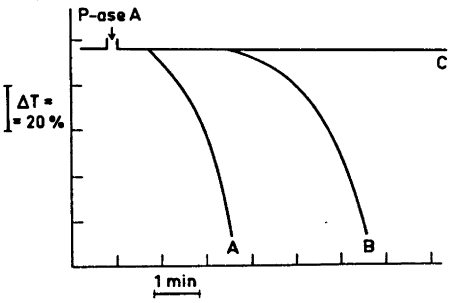

Fig. 3. Inhibition of phospholipase A-induced swelling of mitochondria by ATP. Experimental conditions as in Fig. 1. A. In the presence of 1 mM potassium cyanide alone. B. In the presence of cyanide, 3.3 mM ATP, and 3 μg/ml oligomycin. C. Cyanide plus ATP present.

tion with cyanide or the addition of uncoupling agents restored the action of the enzyme. In the presence of cyanide to suppress respiration, ATP also inhibited the swelling, the inhibition being relieved with oligomycin (Fig. 3). It is evident that the energy state of the mitochondria determines their response to added phospholipase A. This finding might be explained by at least three mechanisms. The energy state might influence the conformation of the mitochondrial membranes, making their phospholipids less vulnerable to enzymatic attack in the energized state. Alternatively, swelling might be prevented by reactivation of the free fatty acids, produced by the activity of phospholipase A, and reacylation of lysophosphatides in the energized state.[7] It is, however, unlikely that under our experimental conditions the rate of this reaction could be sufficient to remove the reaction products of the reaction catalyzed by the added phospholipase A. Thirdly, the enzyme activity *per se* could be inhibited by the removal of calcium, which is known to be an activator of phospholipase A.[8,9] This alternative seems likely, because in the energized state mitochondria are able to accumulate calcium, which is released on deenergisation.[10] By using aequorin, a protein giving luminiscence in the presence of calcium ions, Azzi and Chance[11] have shown that mitochondrial endogenous calcium exists in a dynamic state, showing energy-dependent movements in and out, the external concentration being lowered down to the nM range on energizing the mitochondria. It was therefore of interest to measure the range of calcium concentration needed to activate phospholipase A. Fig. 4 shows that under our conditions, EDTA in the μM range was able to prevent the swelling of mitochondria induced by phospholipase A. Addition of 0.5 μM calcium ions promptly released the inhibition of swelling, caused by 5 μM EDTA. The final concentration of free calcium ions then was probably

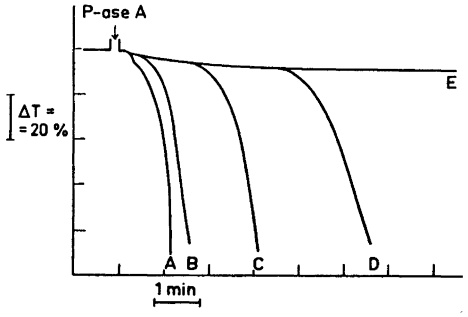

Fig. 4. Effects of EDTA and calcium ions on the phospholipase A-induced swelling of mitochondria. Experimental conditions as in Fig. 1, but 1 mM cyanide present. The concentration of EDTA was 10 μM in E, 2.5 μM in B, 3.7 μM in C, and 5 μM in D. In A, 0.5 μM calcium chloride was present in addition to 5 μM EDTA.

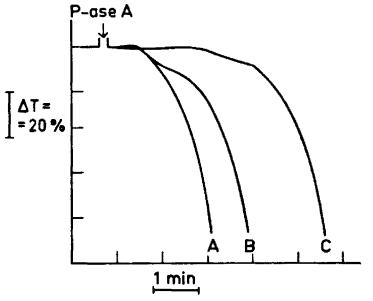

Fig. 5. Effect of lanthanum trichloride on the phospholipase A-induced swelling of mitochondria in the presence of succinate. Experimental conditions as in Fig. 1, except for the amount of mitochondria (1.1 mg protein/ml). A. No succinate added. B. In the presence of 1.5 μM lanthanum trichloride, added 30 sec before succinate, which was added 30 sec before phospholipase A. C. In the presence of succinate alone.

in the nanomolar range. Furthermore, when the energy-dependent uptake of calcium ions was inhibited by lanthanum ions,[12] energisation did not prolong the lag preceding phospholipase A-induced swelling (Fig. 5). In this context it is of less interest that lanthanum ions did not change the biphasic mode of swelling, obtained with energized mitochondria. The data presented above do not exclude conformational changes mediated by changes in the amount and distribution of membrane-bound calcium.

The data presented indicate that phospholipase A-induced swelling may be used to evaluate the energy state of the mitochondria in experimental conditions, where changes in the energy state are reflected in changes in the external calcium concentration, which determines the enzyme activity *per se*. Under these conditions phospholipase A cannot be used to study conformational changes in mitochondrial membranes. We have previously shown [6] that various lipophilic, positively-charged substances inhibit phospholipase A-induced swelling of mitochondria by changing the properties of the mitochondrial membranes, and not by inhibiting enzyme activity *per se*.

Acknowledgements. This study was aided by a grant from the *Sigrid Jusélius Foundation*, and from the *National Research Council for Sciences*, Finland. We are grateful to Mrs. A. Sarasjoki for skillful technical assistance.

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Received May 12, 1970.