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STRUCTURAL CHANGES IN MITOCHONDRIA INDUCED BY UNCOUPLING REAGENTS

THE RESPONSE TO SNAKE-VENOM PHOSPHOLIPASE A

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SUMMARY

Interaction with uncoupling reagents at minimally effective concentrations enhanced the response of freshly isolated rat-liver mitochondria to the action of snake-venom phospholipases. This study corroborated previous findings with proteinases that uncoupling reagents increase the susceptibility of freshly isolated mitochondria to enzymatic attack, as determined by turbidimetric and titrimetric techniques. It is postulated that this effect of uncoupling reagents is due to their binding to protein constituents of the mitochondrial membranes, and a subsequent derangement of the protein–phospholipid organization. The possible relevance of the phenomenon to uncoupling of oxidative phosphorylation is discussed.

INTRODUCTION

In a previous study[1] we have shown that the interaction of isolated rat-liver mitochondria with uncoupling reagents renders the organelles more susceptible to proteolytic enzymes. We postulated that this increased susceptibility results from structural alterations induced by the binding of the uncoupling reagents to specific mitochondrial sites which we believe to be protein[2,3]. Mitochondrial membranes are considered to be composed of protein–phospholipid complexes; therefore, it would be anticipated that a change in the protein conformation would lead to a derangement of the lipoprotein organization. Such a spatial derangement might be identified by an altered susceptibility to phospholipases. In this communication we summarize our observations of the response of mitochondria treated with uncoupling reagents to the action of snake-venom phospholipases.

MATERIALS AND METHODS

Details of the experimental procedures have been described or cited previously[1]. A lyophilized preparation of *Crotalus adamanteus* venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) was used as the source of phospholipase A (EC 3.1.1.4 phos-

phatide acylhydrolase)[4]. Tests with bovine serum albumin, conalbumin, lactalbumin, casein, edestin, and blood hydrolysate as substrates, in the presence or absence of uncoupling reagents, showed that the crude venom was devoid of any significant proteolytic activity at the concentrations used in this study.

*Lysis of mitochondria**

Mitochondria were incubated in the absence and presence of uncoupling reagents under conditions similar to those described previously for the turbidimetric experiments with proteolytic enzymes[1]. Each reaction vessel contained in 4 ml: rat-liver mitochondria (8 mg of protein) suspended in 0.25 M sucrose buffered at pH 8.5 with 0.025 M Tris–HCl. Two samples were incubated simultaneously; a control, and one containing the reagent under study. After 10 min at 25°, 20 μl of snake venom (0.2 %) were added and the samples mixed by gentle inversion. The suspensions were transferred immediately to optical cells and changes in absorbance recorded at 520 mμ in a Cary Model-15 spectrophotometer. Simultaneous recording of changes in absorbance was done by alternately placing the cells in the light path.

In some experiments, lysis of mitochondria was determined also by electrometric titration[1].

RESULTS

Structural studies

As shown in Fig. 1, suspensions of rat-liver mitochondria (control) exposed to *C. adamanteus* venom exhibited, after a short lag period, an abrupt and rapid decrease in turbidity. Other experiments utilizing electrometric titration showed that the lysis of mitochondria was accompanied by the release of free acid groups. Bovine serum albumin (10 μM), but not casein, completely protected the mitochondria from lysis, presumably by binding liberated fatty acids. It has been shown that serum albumin protects mitochondria from the functional alterations induced by other snake venoms[5,6]. Preincubation of mitochondria with a minimal uncoupling concentration of pentachlorophenol (25 μM) markedly enhanced the response to the venom enzyme. It should be emphasized that this concentration of pentachlorophenol alone had no effect on the absorbance of the mitochondrial suspension[1]. Although preincubation was not necessary to elicit a rapid response to snake venom, it was used so that the conditions were similar to those employed in the earlier study with proteinases. Concentrations of pentachlorophenol less than that shown (*e.g.*, 1 μM) also led to some degree of enhancement.

This enhancement of lysis was not unique for pentachlorophenol. Other effective uncoupling reagents were 2,4-dinitrophenol, Dicumarol, carbonyl cyanide *m*-chlorophenylhydrazone, and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (Fig. 2). The latter compound enhanced the lysis of mitochondria at concentrations as low as 10 nM. On the other hand, 2,4,6-trinitrophenol, a reagent devoid of uncoupling properties, was ineffective.

Aged mitochondrial preparations were especially susceptible to the action of snake-venom lipases. Preparations stored at 4° for 24 to 72 h showed an immediate

* In this study, 'lysis' is used as a convenient operational term to indicate that substantial morphological changes occur coincident with the decline in absorbance and release of acid groups.

rapid decrease in turbidity when exposed to snake venom. Consequently, reaction of these stored preparations with pentachlorophenol or with 2,4-dinitrophenol had little influence on the rapid response to the phospholipase.

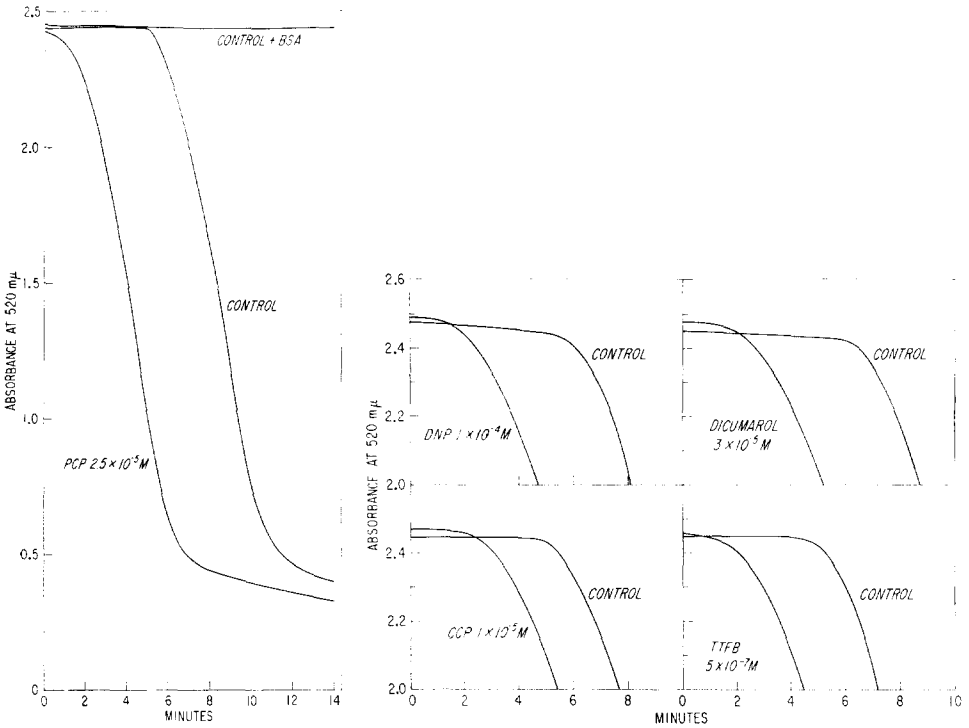

Fig. 1. Effect of pentachlorophenol (PCP) on the lysis of mitochondria by *C. adamanteus* venom. The experiments were conducted as described in the text. The concentration of bovine serum albumin (BSA) was 10 μM.

Fig. 2. The effect of various reagents on the lysis of mitochondria by *C. adamanteus* venom. 2,4-Dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenylhydrazone (CCP), Dicumarol, and 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) were present in the initial incubation mixtures at the concentrations indicated. Only the initial decreases in absorbances are shown. The subsequent decreases were similar to those depicted in Fig. 1.

Functional alterations

Although the effect of snake venom upon functional activities of mitochondria has been studied extensively by others (for a recent review, see ref. 4), it was of interest to determine the functional effects of phospholipases under our experimental conditions. Mitochondria were preincubated in buffered 0.25 M sucrose (pH 8.5) under conditions similar to those used in the turbidimetric experiments. At various times after addition of the venom, samples were removed and assayed polarographically by methods previously described[7]. In general, the sequence of functional loss paralleled that observed with the proteolytic enzymes. Respiratory control, evaluated with a variety of substrates, was the most susceptible. This functional index, which was diminished after the preincubation at pH 8.5, was completely abolished within 2 to 3 min after addition of the venom. Oxidative phosphorylation was abolished

next; the time required for the loss of this activity depended upon the particular substrate used. For example, phosphorylation coupled to the oxidation of α-ketoglutarate was rapidly lost (3 min) followed by that coupled to the oxidation of β-hydroxybutyrate, glutamate, pyruvate + malate, and succinate (15 min).

The non-phosphorylating (oligomycin-insensitive) oxidation of the NAD^+-linked substrates continued until approx. 15 min after addition of the venom. As was observed with the proteinases, this decline in oxidation apparently was preceded by the loss of electron carriers because the addition of NAD^+ or cytochrome *c* could stimulate the respiration of the treated mitochondria for a short time. However, the enzyme complexes which catalyze the oxidation of succinate and NADH were extremely resistant to the action of phospholipase. Their activity could be detected 90 min after treatment of mitochondria with venom.

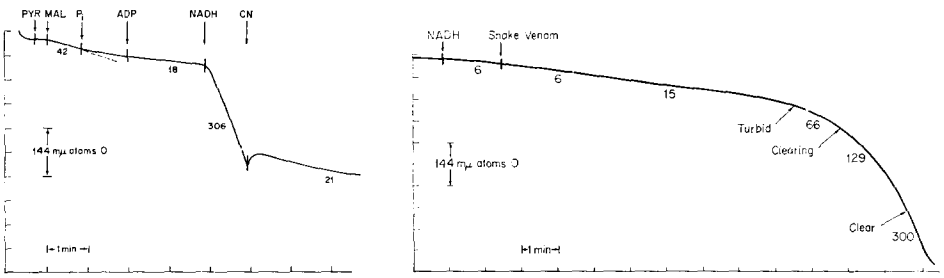

Fig. 3. Polarographic assay of the effects of snake venom on mitochondrial function. Mitochondria (16 mg of protein) were suspended in 8 ml of 0.25 M sucrose–0.025 M Tris buffer (pH 8.5). After 10 min incubation at 25°, 80 μg of *C. adamanteus* venom were added and 1.5-ml samples were removed for polarographic assay. In the experiment illustrated, the sample was removed 12 min after addition of the venom. The additions were: 10 μmoles of pyruvate (PYR), 10 μmoles of malate (MAL), 10 μmoles of P_i, 0.5 μmole of ADP, 1.2 μmoles of NADH and 1.5 μmoles of KCN (CN). The numbers indicate the rate of oxidation in natoms/min.

Fig. 4. Effect of *C. adamanteus* venom on the oxidation of NADH by mitochondria. Mitochondria (4 mg of protein) were suspended in 1.5 ml of 0.25 M sucrose–0.025 M Tris buffer (pH 8.5). At the times indicated, 1.2 μmoles of NADH and 20 μg of *C. adamanteus* venom were added. 'Clearing' refers to the onset of a rapid decrease in the turbidity of the suspension. The numbers indicate the rate of oxidation in natoms/min.

Figs. 3 and 4 illustrate some results of these polarographic studies. It may be seen that with mitochondria treated with venom the oxidation of NAD^+-linked substrates (or succinate, not shown) was not stimulated by the addition of P_i or ADP (Fig. 3). On the contrary, the non-phosphorylative respiration was inhibited by the addition of phosphate and phosphate acceptor. A similar inhibition has been observed by ZIEGLER *et al.*[6] with mitochondria treated with the venom of *Bungarus fasciatus*. The explanation for this 'reverse acceptor control' (ref. 8) remains obscure. Fig 3 also shows that the lysed mitochondria oxidized added NADH at a rapid rate after the oxidation of NAD^+-linked substrates was abolished. Cyanide was inhibitory.

Fig. 4 illustrates the correlation of the onset of the rapid rate of oxidation of NADH with the sudden clearing of the mitochondrial suspensions. The oxidation of NADH in these experiments was sensitive to Amytal.

DISCUSSION

Various experimental techniques have been used to study the relationship of structural and functional states in isolated mitochondria[9–11]. For example, it has been shown that exposure of rat-liver mitochondria to snake-venom phospholipase A leads initially to an uncoupling of oxidative phosphorylation, and ultimately, to the cessation of oxidation of various substrates with a concomitant derangement of mitochondrial structure. These effects have been considered to be the result of alteration or removal of phospholipids[12] leading to changes in the spatial configuration of the remaining components[5].

The present study, as well as a previous one[1], demonstrates that the response of mitochondria to lytic enzymes can be an effective means of determining changes in the molecular organization of the phospholipids and proteins which comprise the mitochondrial membranes. This technique is based upon the widely held assumption that an altered response to lytic enzymes reflects an altered conformation of the substrate for these enzymes[13].

In this communication we have shown that the reaction of mitochondria with uncoupling reagents enhances the onset of their lysis by snake-venom phospholipases. Undoubtedly, free fatty acids, liberated by the hydrolysis of phospholipids, contribute to dissolution of the mitochondria. Uncoupling reagents alone may release fatty acids. However, it is doubtful if this occurred to any significant extent in the present experiments as there was no decrease in the absorbance of the mitochondrial suspensions when incubated with these reagents alone. Alternatively, it is conceivable that the uncoupling reagents may have inhibited the incorporation of liberated fatty acids into phospholipids resynthesized during the lag phase of mitochondrial lysis induced by the phospholipases. This also appears unlikely as the resynthesis of mitochondrial phospholipids requires exogenous ATP and is accompained by contraction of the swollen organelles[14].

The mitochondrion is considered to be a macromolecular array of phospholipid–protein complexes. Therefore, the results with proteinases presented previously[1], and with the snake-venom phospholipases shown here, can be interpreted as indicating that the arrangement of both major components of the complex are altered by interaction with uncoupling reagents. We believe that the observed synergism between uncoupling reagents and lytic enzymes is a result of conformational changes induced by the interaction of the uncoupling reagents with proteins of the mitochondrial membranes[2,3].

It should be emphasized that the only functional derangement observed with the concentrations of reagents used in this study is uncoupling of oxidative phosphorylation. Significantly, 2,4,6-trinitrophenol, a reagent which interacts with mitochondria but does not uncouple phosphorylation[3], had no effect on the lysis of mitochondria by snake venom. Therefore, we envisage that it is the interaction of uncoupling reagents with protein sites at the locus of energy conservation that induces structural alterations which lead to loss of coupled phosphorylation.

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