ON THE DIFFERENCES IN THE SENSITIVITY OF COUPLING SITES OF SUBMITOCHONDRIAL PARTICLES TO PHOSPHOLIPASES A, C AND D*

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1. Introduction

Phospholipases A, C and D are widely used for studies on the structure of membranes of mitochondria [1–3], erythrocytes [4,5] and microsomes [6]. Information about the arrangement of phospholipids in the membrane can be obtained by elucidating the accessability of certain parts of phospholipid molecules to phospholipase attack. The sensitivity of various respiratory chain segments to cobra venom phospholipase A has been studied in this laboratory [7]. The data of the present work concern the sensitivity of various energy-linked functions of submitochondrial particles to phospholipases A, C and D. These data testify to the I and IV coupling sites, being of maximal sensitivity to phospholipase A, while coupling sites II and/or III are the most sensitive to phospholipases C and D.

2. Materials and methods

Ultrasonic submitochondrial particles, ETP_H (Mg^{2+}, Mn^{2+}), were obtained from beef heart heavy mitochondria according to Beyer [8].

2.1. *Incubation conditions*

Submitochondrial particles were incubated with phospholipases C and D at 30°C in 50 mM sodium acetate (pH 6.5), 0.25 M sucrose and 2 mM $MgSO_4$. The concentration of submitochondrial particles was 2.0 mg/ml, the concentrations of phospholipases C and D – 0.1–0.2 mg/mg particle protein. During the phospholipase C treament the incubation medium als contained 1 mM $CaCl_2$, and during the phospholipase D treatment – 10–40 mM $CaCl_2$.

The incubation of the particles with phospholipase A was carried out as in the previous work [9].

2.2. *Assays*

(1) The rate of ADP phosphorylation coupled to succinate oxidation was measured at 36°C in a medium containing 0.25 M sucrose, 20 mM Tris–HCl (pH 7.5), 10 mM potassium phosphate (pH 7.5), 3 mM $MgSO_4$, 40 mM glucose, 0.5 mM EDTA, hexokinase (100 μg per ml), glucose-6-phosphate dehydrogenase (20-30 μg per ml), 2.0 mM ADP, 0.5 mM $NADP^+$, rotenone (0.16 μg per ml), 8 mM succinate, 5 mM AMP, BSA (0.8 mg per ml) and submitochondrial particles (80 μg per ml). AMP was added to the medium to suppress adenylate kinase activity. Aliquots of the particle suspension (0.1 ml) were drawn from the incubation mixture and transferred to the assay medium, after which residual adenylate kinase activity was recorded. Then succinate was introduced to the assay medium. The rate of ADP phosphorylation was corrected for adenylate kinase activity. The reaction was followed spectrophotometrically at 340 nm.

(2) The ATP-dependent transhydrogenase activity and the rate of ATP-dependent NAD^+ reduction by

* Phospholipase A from *Crotalus terrificus* venom, phospholipase C from *Clostridium welchii* and phospholipase D from cabbage were purchased from Calbiochem, Inc.

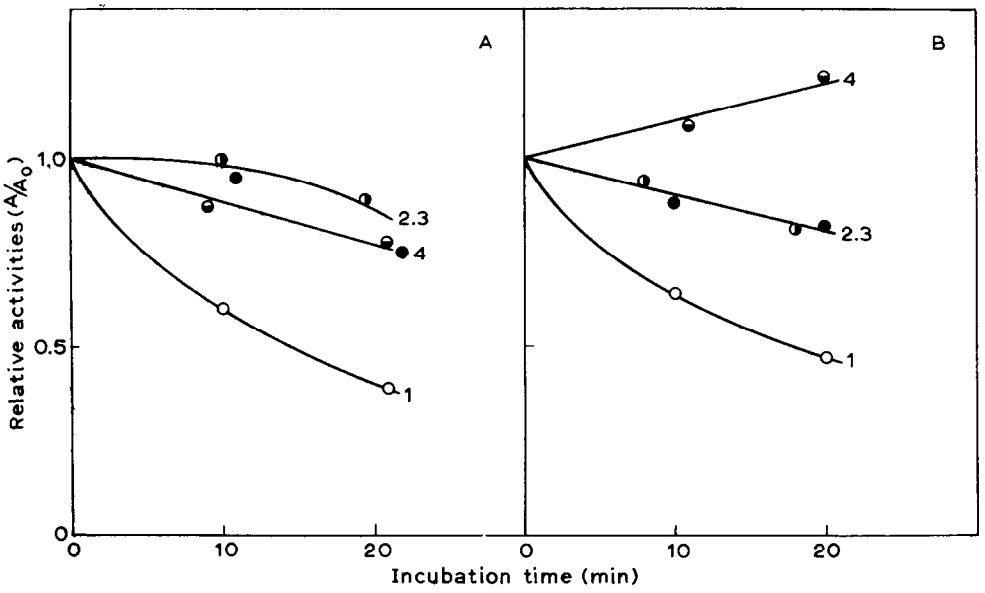

Fig. 1. Sensitivity of various functions of submitochondrial particles to phospholipases C and D. A) Effects of phospholipase C: 1, Phosphorylating activity; 2, ATP-dependent NAD^+ reduction by succinate; 3, ATP-dependent transhydrogenase; 4, Succinate oxidase. The incubation medium contained 0.1 mg phospholipase C per mg particle protein. 1 mM $CaCl_2$ and 2.0 mg particle protein per ml. B) Effects of phospholipase D: 1, Phosphorylating activity; 2, ATP-dependent NAD^+ reduction by succinate; 3, ATP-dependent transhydrogenase; 4, Succinate oxidase. Concentration of phospholipase D was 0.15 mg per mg particle protein, $CaCl_2$ – 30 mM and particle protein – 2.0 mg/ml.

succinate were measured at 30°C as in the previous work [9].

(3) Succinate oxidase activity was determined at 36°C as described elsewhere [10].

3. Results and discussion

3.1. *Phospholipases C and D.*

Fig 1A demonstrates that it is the ADP phosphorylation coupled to succinate oxidation in submitochondrial particles that is the most sensitive to the action of phospholipase C (curve 1). The rates of ATP-dependent NAD^+ reduction by succinate, ATP-dependent transhydrogenase and succinate oxidase reactions under the same conditions decrease significantly slower (fig. 1, curves 2–4).

Similar results were obtained for phospholipase D (Fig. 1B). In this case, too, the most rapidly decreasing was the rate of ADP phosporylation (curve 1). As to ATP-dependent NAD^+ reduction by succinate and ATP-dependent transhydrogenase, they were suppressed substantially slower (curves 2, 3) and succinate oxidase was even slightly activated (curve 4).

A special experiment gave evidence to that the products of phospholipid cleavage in submitochondrial particles by phospholipases C and D did not detectably inhibit phosphorylation of ADP. For this reason the changes observed should be ascribed to the damage to the fine structure of some regions of the membrane.

3.2 *Phospholipase A.*

The results obtained for phospholipase A from *Crotalus terrificus* venom were of another kind. As has been indicated in our previous work [9], reversed electron transfer and energy-dependent transhydrogenase are highly sensitive to phospholipase A. In addition to these data, the results depicted in fig. 2 show a relatively high resistance of ADP phosphorylation to phospholipase A. It follows from fig. 2 that during

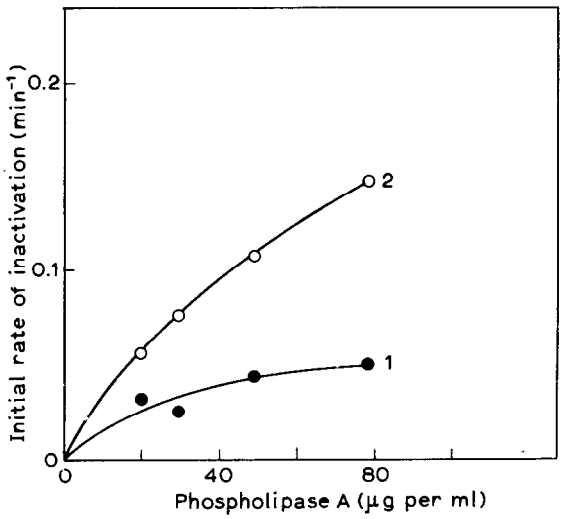

Fig. 2. Comparative sensitivity of ADP phosphorylation and reversed electron transfer to phospholipase A. 1, ADP phosphorylation; 2, ATP-dependent NAD^+ reduction by succinate. Particles (2.0 mg/ml) were incubated at 30°C in the medium containing 50 mM Tris–HCl (pH 7.5), 0.25 M sucrose and 3 mM $MgSO_4$.

treatment of the particles with phospholipase A this function is impaired less readily than reversed electron transfer. Since all measurements were carried out in the presence of excess amounts of serum albumin, the effects described were most probably caused not by the uncoupling action of the products of phospolipid cleavage by phospholipase A, but by the damage to the fine structure of certain regions of the membrane.

Comparing the results obtained for phospholipases C and D with those for phospholipase A, one may conclude that the coupling sites display different sensitivity to the above-mentioned phospholipases. As to phospholipase A, it mainly impairs the I and IV* coupling sites (see fig. 2, and ref. [9]), affecting the II and III coupling sites and the ATP-synthesizing system less profoundly. On the contrary, phospholipases C and D, apparently, destroy cliefly the II and/or III coupling sites (see fig. 1 A,B). Indeed, the decrease in the rate of ADP phosphorylation induced by the action of phospholipases on submitochondrial particles could be due to: (1) degradative changes in the succinate oxidase system; (2) inactivation of the ATP-synthesizing system (if really existent**) and (3) destruction of certain coupling sites. As to succinate oxidase, it was not substantially inactivated in our experiments (see fig. 1 A,B). It could be supposed that the link responsible for the high sensitivity of ADP phosphorylation to phospholipases C and D is the ATP-synthesizing system. But in this case it is not clear why ATP-dependent NAD^+ reduction by succinate is less sensitive to the phospholipases indicated than phosphorylation of ADP. Here it must be remembered that the agents directly affecting the ATP-synthesizing system (such as oligomycin [13] and trypsin [14]) hinder ADP phosphorylation and reversed electron transfer [13,14] to the same extent. Hence it can be suggested that the effects of phospholipases C and D depicted in fig. 1 A,B were caused mainly by disturbance of II and/or III coupling sites. The insignificant decrease in the rates of ATP-dependent NAD^+ reduction by succinate and ATP-dependent transhydrogenase reaction prompts one to believe that I and IV coupling sites of submitochondrial particles are of little sensitivity to phospholipases C and D.

Hence, phospholipase A that splits off the hydrophobic 'tails' of phospholipid molecules and phospholipases C and D, destroying the polar 'heads' thereof, display a certain selectivity for different coupling sites of submitochondrial particles.

Firstly, this could be ascribed to differences in the substrate specificity of phospholipases A, C and D. In this connection it must be pointed out that phospholipase C from *Clostridium welchii* and phospholipase D from cabbage, when acting on mitochondrial membrane and phospholipid micelles rapidly cleave phosphatidyl choline, destroying phosphatidylethanolamine more slowly and leaving cardiolipin unaffected [3,15,16]. Contrariwise, phospholipase A from *Naja naja* venom splits phosphatidylethanolamine more rapidly than phosphatidylcholine. As to cardiolipin, it is slowly hydrolyzed by phospholipase A from *Naja naja* and is almost unaffected by phospholipase A from *Crotalus sp.* venoms [3,15].

* According to the terminology of Skulachev [11] the IV coupling site refers to the energy-linked transhydrogenase.

** See, for instance, refs. [12,22].

The data mentioned above, together with the results of the present work, suggest that phosphatidylcholine and phosphatidylethanolamine are of unequal importance for the functioning of I and IV coupling sites on the one hand, and coupling sites II and/or III, on the other hand. Indirect evidence for this point of view are the data of Racker's laboratory, which demonstrate a strict dependence of oxidative phosphorylation efficiency on phospholipid composition during reconstitution on the first and third segments of oxidative phosphorylation system [17,18].

Secondly, it may well be assumed that the differences in sensitivity of certain coupling sites to the phospholipases mentioned reflect the structural specialities of the corresponding membrane regions. In particular, it can be supposed that phospholipid molecules are dissimilarly arranged in I and IV coupling sites on the one hand, and in II and/or III sites, on the other hand, this resulting in the different accessability of phospholipids in the sites indicated to phospholipases A, C and D.

The third suggestion is that the accessability of the bonds hydrolyzed in phospholipids is approximately similar in different membrane regions; but of primary importance for the maintenance of the structure of coupling sites II and/or III are the electrostatic interactions disturbed due to the action of phospholipases C and D, while in the case of I and IV coupling sites the most important are the hydrophobic interactions disturbed by phospholipase A.

Finally, it is possible to suggest that coupling sites are localized in membrane regions bearing different local surface charges, the sign and magnitude of which may significantly influence the rate of phospholipid cleavage (see, for example, refs. [19–21]).

It is now impossible to choose among these suppositions. Nevertheless, no matter what the nature of the differences in the sensitivity of the coupling sites to phospholipases is, their cause obviously lies in the peculiarities of the architecture of the corresponding membrane regions.

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