# ROLE OF PHOSPHATIDYLSERINE AND PHOSPHATIDYLCHOLINE IN THE DICYCLOHEXYLCARBODIIMIDE-INDUCED INHIBITION OF MITOCHONDRIAL ATPase

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Summary. Mixed phospholipids, purified phosphatidylcholine and phosphatidylserine restored the ATPase activity of submitochondrial particles pretreated with dicyclohexylcarbodiimide (DCCD). Pretreatment of mixed phospholipids with DCCD resulted in preparations which were able to transfer the inhibitor to submitochondrial particles. Phosphatidylcholine alone was fully effective.Phosphatidylserine, which apparently forms a stable bond with DCCD, was inactive.These findings suggest different functions of individual phospholipids in the DCCD-induced inhibition. The observation that phosphatidylserine displays unusual high activity in the restoration of ATPase activity in phospholipid-depleted submitochondrial particles indicates that it plays an important role in the activation of mitochondrial ATPase and deserves attention as costituent of the final receptor site for DCCD.

It was shown previously (1-3) that the inhibition produced by DCCD and oligomycin on rat-liver mitochondrial ATPase and by oligomycin on calf-heart $Na^+$-$K^+$-stimulated ATPase can be prevented by phospholipids. The antagonism has been taken as indication of a high affinity of the inhibitors for the phospholipids which constitute a great part of the lipid portion of these membranous fragments. Direct or indirect alteration of the functional protein could be produced after distribution of the inhibitory compounds in the phospholipids. A recent study (4) on the effect of carbodiimides with different solubility in lipids on ATPase from Streptococcus foecalis, is in close agreement with this hypothesis.

In this report a more direct evidence for the partecipation of phospholipids in the inhibitory effect of DCCD on particulate mitochondrial ATPase from rat-liver is presented.

Materials and Methods. Submitochondrial particles (SMP) were obtained from rat-liver mitochondria according to Kielley and Bronk (5). SMP were depleted of phospholipids by the procedure devised by Kagawa and Racker (6) for the

preparation of $CF_0 \cdot F_1$. Trypsin treatment was omitted. Phospholipids were prepared and used as described (1). In all cases it was assumed that 1 µg of totale phosphorus is equivalent to 25.3 ug of phospholipid (7). In order to reduce the inhibition of ATPase by the aggregates of phosphatidylserine, which was observed earlier (1), the sonication in this case was made after neutralization of the phospholipid with 0.5 M TRIS.HCl pH 8.0.

Preincubation of phospholipids with DCCD was made as follows : 2.5 mg phospholipids were equilibrated at 37° in 0.5 ml of 50 mM TRIS.HCl pH 7.5, 2.5 mM $MgCl_2$, 125 mM sucrose, 0.5 mM EDTA. 10 microliters of ethanol containing 100 nmoles DCCD were added and the sample incubated 10 min at 37° unless otherwise stated. Control samples with phospholipids plus ethanol alone or with DCCD in the absence of phospholipids were run simultaneously. The samples were filtered through a 4 ml column of Sephadex G-200 (Pharmacia, Sweden) packed and equilibrated four hours at room temperature with 5 mM TRIS.HCl pH 7.5. Elution was performed using the diluted TRIS.HCl solution and fractions of 1.0 ml were collected. Aliquots were assayed for phospholipid phosphorus and for inhibitory effect on ATPase activity of SMP. When required, bovine serum albumine was used instead of phospholipids and the same procedure was followed. ATPase activity was measured at 37° under the conditions outlined in the legend to Table I, proteins by the procedure of Lowry et al. (8).

Results. In Table I it is seen the antagonism produced by several phospholipids on SMP pretreated with DCCD at 37° and 0°. Pooled mitochondrial phospholipids from rat-liver, purified phosphatidylcholine and phosphatidylserine from bovine brain produced substantial removal of DCCD-induced inhibition, synthetic dipalmitoyl-lecithin and bovine serum albumine were less active.

It was previously reported (9,10) that washing with phospholipids submitochondrial particles from ox-heart incubated with the inhibitor, was not sufficient to remove DCCD and restore inhibited activities.It was concluded that DCCD was irreversibly bound. However, the effect of phospholipids in these different conditions indicates that the stage of irreversible effect develops gradually and during this time partial redistribution of DCCD with the external phospholipids can take place. This

TABLE I

Effect of phospholipids on DCCD-treated submitochondrial particles (SMP)

Each tube contained in a final volume of 1.0 ml 50 mM TRIS.HCl pH 7.5, 2.5 mM $MgCl_2$, 3.0 mM ATP.TRIS pH 7.4, 50 mM sucrose, 0.2 mM EDTA, 1 % (v/v) ethanol containing or not DCCD, SMP from rat-liver 20-30 µg of protein. The reaction was started with SMP. When SMP had to be treated with DCCD, the reaction was started by simultaneous addition of ATP and phospholipids after the time indicated. The complete system was incubated 20 min at 37° and the reaction was terminated by addition of trichloroacetic acid (final concentration, 10 %).

| Exp. N° | Additions | Treatment of SMP with DCCD | µmoles ATP split/mg/h without DCCD | µmoles ATP split/mg/h with 0.35 nmoles DCCD | % inhib. |
|---|---|---|---|---|---|
| 1 | - | none | 170 | 18 | 89.4 |
| | 0.9 mg RLMPL | none | 163 | 128 | 21.5 |
| | id. | 2 min 37° | 151 | 87 | 42.5 |
| | id. | 5 min 37° | 155 | 61 | 60.6 |
| | id. | 10 min 0° | 158 | 91 | 42.5 |
| 2 | - | 10 min 0° | 158 | 57.5 | 63.6 |
| | 0.6 mg LEC. | id. | 153 | 108 | 29.4 |
| | 0.9 mg P.SER. | id. | 110 | 105 | 4.5 |
| | 0.9 mg DPL | id. | 148 | 79 | 46.6 |
| | 0.95 mg BSA | id. | 155 | 73 | 52.9 |
| 3 | - | 45 min 0° | 121 | 24 | 80.2 |
| | 0.9 mg LEC | id. | 135 | 56 | 58.5 |
| | 0.5 mg P.SER | id. | 81 | 56 | 30.8 |
| | 0.9 mg BSA | id. | 113 | 24 | 78.8 |

RLMPL = rat-liver mitochondrial phospholipids; LEC. = bovine brain lecithin; P.SER = bovine brain phosphatidylserine; DPL = dipalmitoyl (synthetic)lecithin; BSA = bovine serum albumine.

suggests the transient formation of a reversible bond between DCCD and some component of the enzyme-phospholipids. The removal of DCCD can be facilitated by an exchange between the external and the endogenous phospholipids (11).

If this is correct, the reverse effect should also be demonstrated : phospholipids enriched with DCCD could become a carrier of the inhibitor. The experiment of Fig. 1 gives support to this possibility. To exclude that unbound DCCD would have been added together with the DCCD-pretreated phospholipids these were filtered through Sephadex. Inhibition of ATPase was not produced by the fractions collected from the column with DCCD alone, showing that the inhibitor remained in the column. By contrast, phospholipids were

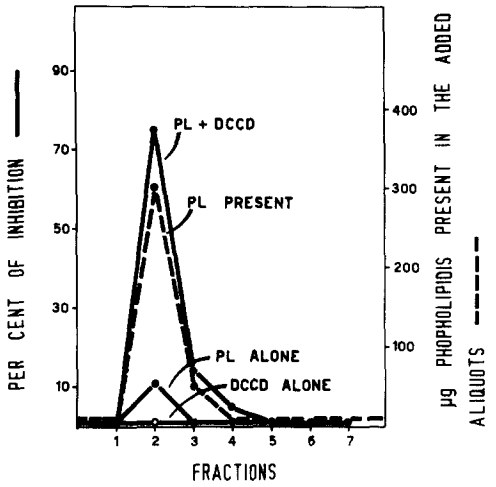


Fig. 1 Transport of DCCD by phospholipids. Bovine brain phospholipids (PL) were preincubated with DCCD and filtered through G-200 Sephadex under the conditions outlined in "Materials and Methods". 0.2 ml aliquots of the fractions were assayed for phospholipid phosphorus and ability to induce inhibition of ATPase activity of SMP from rat-liver. Conditions for the measurement of ATPase as described in Table I.

excluded and recovered mainly in the second fraction. Phospholipids pretreated with DCCD retained the inhibitor and were able to transfer it to SMP as revealed by the strong inhibition produced in coincidence with the efflux of phospholipid phosphorus.

Phospholipids from mitochondria and bovine brain were equally active as carriers of DCCD. Among individual purified phospholipids, phosphatidylcholine from bovine brain was found highly effective whereas synthetic dipalmitoyl-lecithin was only slightly active and the acidic phosphatidylserine from bovine brain totally inactive. Bovine serum albumine, a well known drug-binder protein, showed only negligible activity, which was not always reproducible.

The reason for the ineffectiveness of phosphatidylserine was found in the experiment of Table II. Preincubation of DCCD with this phospholipid resulted in a progressive decrease of the inhibitory effect, which on the contrary was enhanced when the preincubation was made with phosphatidylcholine and serum albumine. Presumably a stable bond is formed between phosphatidylserine and DCCD which preclude the inhibitor to be transferred to the

TABLE II

Inactivation of DCCD by phosphatidylserine

Bovine serum albumine, bovine brain lecithin and bovine brain phosphatidylserine were incubated with 100 nmoles DCCD in the conditions described in "Materials and Methods", Sephadex filtration was omitted. After the time indicated 10 µl aliquots (corresponding to 2 nmoles DCCD) were assayed for inhibitory effect on SMP in the conditions described in Table I.

| Additions to DCCD during preincubation | Percent of inhibition by 2 nmoles DCCD after : | | |
|---|---|---|---|
| | 10 min | 20 min | 30 min |
| none | 70 | 73 | 66.5 |
| bovine brain lecithin | 90 | 90 | 93.5 |
| bovine brain phosphatidylserine | 55 | 35.5 | 23 |
| bovine serum albumine | 86.5 | 86.5 | 90 |

enzyme preparation. From this result the possibility that phosphatidylserine is part of the final receptor for DCCD on SMP is worth to be considered.

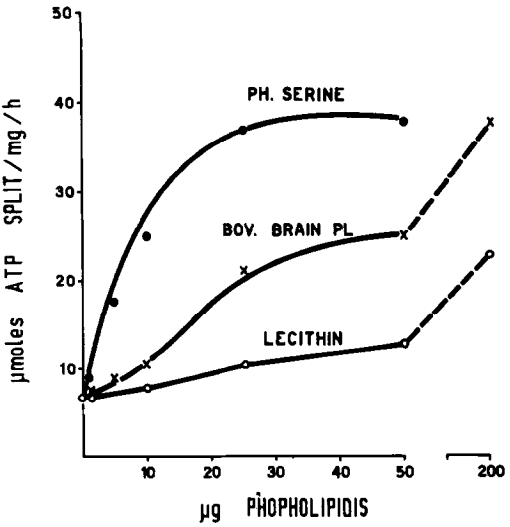


Fig. 2 Reactivation of cholate-treated submitochondrial particles by phospholipids. Pooled bovine brain phospholipids, bovine brain phosphatidylserine and phosphatidylcholine (lecithin) were incubated 5 min at 37° in the amount indicated, with 120 µg of protein cholate-treated SMP in 0.2 ml of 0.25 M sucrose, 10 mM, TRIS.HCl pH 7.5, 1 mM EDTA. The systems were completed and the ATPase activity was determined as described in Table I.

In Fig. 2 it is seen that phosphatidylserine was far more active than phosphatidylcholine and pooled bovine brain phospholipids in the reactivation of SMP which were 90 % depleted of phospholipids by cholate treatment. The activity of phosphatidylserine was surprisingly high since half-maximum effect was attained at about 60 μg/mg protein. This is in accordance with the low content of this phospholipid in the mitochondria.

Discussion. Depending on the experimental conditions phospholipids can be antagonist of DCCD-induced inhibition or carriers of the inhibitor. These two properties are not equally distributed among individual phospholipids. The neutral phospholipid phosphatidylcholine exhibits both effects since it is able to bind reversibly the inhibitor. Saturation of paraffin chains reduces this activity. With the acidic phospholipid, phosphatidylserine only the antagonistic effect can be put in evidence presumably because it forms a stable bond with the inhibitor. The different behavior of the two phospholipids would justify distinct functions in the process of the DCCD-induced inhibition. Phosphatidylcholine appears suitable to promote the initial distribution of DCCD within the membranous fragments and the transfer to the final receptor. Phosphatidylserine can be a component of the final DCCD-receptor site. Its high activity in the reactivation of phospholipid-depleted SMP is consistent with this possibility and is suggestive of an important role of this phospholipid in the activation of mitochondrial particulate ATPase.

The structure of phosphatidylserine is appropriate to fulfill the role of receptor for DCCD. It contains a carboxyl group which is in the context of a molecule with large hydrophobic region. Carbodiimides react preferentially with nonionized carboxyl groups and it has been suggested (4) that the receptor for DCCD should include a carboxyl group whose ionization at physiological pH is prevented by a hydrophobic environment. Some difficulties arise from the observation that phosphatidylserine is required for the operations of $Na^+$-$K^+$-stimulated ATPase (12-14), which is little affected by DCCD (3). However a different dislocation of phosphatidylserine in the two ATPases would explain dissimilar reactivity of the carboxyl group. Oligomycin, which has a site of action very close, if not identical, to that of DCCD, does not require irreversible bond with functional groups to produce inhibition.

Accordingly, it affects both ATPases. Beechey et al. (15) reported the isolation of proteolipids retaining large part of the radioactivity after preincubation of submitochondrial particles from ox-heart with labelled DCCD. The presence of phospholipids in this fraction was not excluded.

If the proposal of an essential role of phosphatidylserine in mitochondrial ATPase will be substantiated it would be possible to establish an interesting analogy with the $Na^+$-$K^+$-stimulated ATPase. Presumably the molecule of phosphatidylserine is particularly suited to satisfy the requirements of membrane associated ATPases which have in common, among other properties, the capacity to use chemical energy to build ion-gradients across biological membranes.

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