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Effects of phospholipases C and D on ordering of channel proteins in the mitochondrial outer membrane

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The effects of phospholipases C and D on the state of order of the channels in the outer membranes of *Neurospora* mitochondria have been investigated by negative-stain electron microscopy and optical diffraction. Unlike the situation with phospholipase A_2, treatment of the isolated membranes with phospholipase C or D does not induce crystallization of the channels in the membrane plane. Furthermore, treatment of already-formed periodic arrays of outer membrane channels with either phospholipase C or D causes loss of long-range order in the arrays. The latter result suggests that zwitterionic phospholipids may play an important role in stabilizing the periodic arrays of the channel-forming protein in this membrane.

Introduction

The principal protein component of outer membranes of *Neurospora* mitochondria is the 31 kDa pore-former, called VDAC or mitochondrial porin [1–4]. Treatment of these membranes with low levels of soluble phospholipase A_2 during dialysis against low-salt buffer induces crystallization of the pore protein in the membrane plane [5,6]. The concomitant decrease in surface area and increase in buoyant density of the mitochondrial outer membranes suggest that ordering of this integral protein is associated with removal of phospholipids from the membranes, presumably after their hydrolysis to lysophospholipids and free fatty acids by phospholipase A_2 [5,6]. Earlier observations of periodic arrays of the pore protein on untreated outer membranes from *Neurospora* mitochondria [1,4] suggest that endogenous mitochondrial phospholipase A_2 activity [7,8] may be effective in inducing ordering of the channels. Such phospholipase A_2-induced crystallization of mitochondrial outer membrane channels may have functional implications. For example, it has recently been shown that clustering of acetylcholine receptors alters their channel kinetics [9]. However, at present, our primary interest in this phenomenon of phospholipase-induced ordering of membrane proteins is a practical one, i.e., the preparation of large two-dimensional crystalline arrays for low-dose electron microscopy and image analysis [10].

The phospholipase A_2-dialysis technique has been successfully applied to the planar crystallization of at least one other membrane protein, the Ca^{2+}-ATPase of sarcoplasmic reticulum [11]. The relative speed and simplicity of the technique makes it an attractive starting point when attempting to crystallize proteins present in large concentration in native or reconstituted membranes.

In this report, we examine the effects of two

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other soluble phospholipases on the state of organization of the channels in mitochondrial outer membranes, phospholipases C and D. These enzymes act at the polar head groups of phospholipids, resulting in formation of either diacylglycerol (phospholipase C) or phosphatidic acid (phospholipase D). Negative-stain electron microscopy and optical diffraction are used to address two basic questions: (1) do these enzymes, like phospholipase A_2, induce ordering of the channels in native *Neurospora* mitochondrial outer membranes; and (2) what effect do the enzymes have on already-formed periodic arrays of the outer-membrane channel protein?

Materials and Methods

Mitochondrial outer membrane isolation

Mitochondria were isolated from late-exponential phase protoplasts of a slime mutant of *Neurospora crassa* by differential centrifugation [1,6]. Outer membranes were isolated by density gradient centrifugation of hypoosmotically swollen mitochondria [1,6]. Purity of the outer-membrane fractions obtained is routinely assessed by SDS-polyacrylamide gel electrophoresis (see Fig. 3 in Ref. 1) and negative-stain electron microscopy [1].

Phospholipase-dialysis treatment

The basic experiment described below was done twice in its entirety, and repeated in parts (with variations) several more times. Immediately after collection on sucrose step gradients, the outer mitochondrial membrane fraction (containing approx. 0.3 mg protein) was diluted to 140 ml with buffer 1 (1 mM Tris-HCl/0.25 mM EDTA (pH 7.5)). This suspension was divided into four 35 ml aliquots which were pipetted into standard cellulose dialysis bags (Molecular weight cutoff approx. 10000; prewashed with hot 50 mM Na_2CO_3/10 mM EDTA). To the different bags (specimens 1–4) were added (1) nothing, (2) 60 units bee venom phospholipase A_2, (3) 65 units *Clostridium* phospholipase C, or (4) 200 units peanut phospholipase D. (Each enzyme was obtained as a lyophilized powder of the highest available specific activity from Sigma Chemical Co., St. Louis, MO. The powders were dissolved in a small volume of 50% (v/v) aqueous glycerol just prior to use. Phospholipase activity units are defined as that amount of enzyme which hydrolyzes 1 μmol phospholipid/min at standard conditions, which vary somewhat for each enzyme.) The membrane suspensions were dialyzed 18 h at 4°C against 1000 vol. buffer 1, after which they were transferred to centrifuge tubes and pelleted (60000 × *g*, 90 min). Each membrane pellet was resuspended in 0.15 ml buffer 1, of which 0.05 ml was used for negative-stain electron microscopy (see below). The remainder of each specimen 2–4 (now called 2′–4′) was rediluted with buffer 1 to 20 ml. Both specimens 3′ and 4′ were transferred to dialysis bags, to which were added 60 units phospholipase A_2. Specimen 2′ was divided into two 10 ml aliquots, 2′a and 2′b, which were also transferred to dialysis bags and to which were added, respectively, 65 units phospholipase C and 300 units phospholipase D. Specimens 2′a–4′ were dialyzed overnight against 1000 vol. buffer 1, pelleted (as above) and resuspended in 0.05–0.1 ml buffer 1 for electron microscopic specimen preparation.

In experiments to determine the time-course of the effects of phospholipases C and D on mitochondrial outer membrane structure, three 0.1 ml aliquots of a membrane suspension (approx. 0.25 mg protein/ml buffer 1) containing either (1) nothing, (2) 3 units phospholipase C, or (3) 3 units phospholipase D were transferred to the wells of a microdialyzer unit (Bethesda Research Labs, Bethesda, MD). Small (10 μl) aliquots were removed for electron microscopy at intervals over several hours while the specimens dialyzed continuously at room temperature against buffer 1. Because of the greater membrane concentration in these specimens (approx. 250 vs. 2 μg membrane protein/ml), the suspensions could be used directly for negative-stain specimen preparation (i.e., without first concentrating the membranes by centrifugation). Because the relative volumes of the phospholipase C and D solutions to those of the membrane suspensions were much greater than in the previous experiments, enzyme solutions were first dialyzed against 250 ml buffer 1 (to remove buffer salts contained in the commercial enzyme preparations) prior to mixing with the membranes. The enzyme concentrations in these experiments

(30 units/ml) were in the same range as those used in the previous experiments, although the amount of enzyme to that of membrane was lower (approx. 120 vs. 2600–12 000 units/mg membrane protein). The decreased enzyme-to-substrate ratio in the later experiments was offset by increasing the temperature of the reaction (from 4 to 20°C).

Electron microscopy

Membrane suspensions treated as described above were deposited on glow-discharged carbon/formvar films and negatively stained with 1% uranyl acetate as described in detail elsewhere [1]. These specimens were examined in the electron microscope (Philips EM301 or EM420T, 100 kV) within a few hours of preparation. Images were recorded on SO-163 film (Kodak, Rochester, NY) at instrument magnifications of 20 000–50 000 ×.

Optical diffraction analysis

Optical diffraction patterns of membrane images were recorded directly from electron microscopic negatives. Circular areas on the negatives were irradiated with coherrent radiation from an He-Ne laser (Jodon Engineering Assoc., Ann Arbor, MI) and the diffraction patterns were recorded on Panatomic-X film (Kodak, Rochester, NY).

To quantitate disordering effects of certain treatments (see Fig. 3), optical diffraction patterns were recorded from images taken at the same magnification (23 000 ×) using a fixed circular aperture corresponding to an area 200 nm in diameter on each membrane. (Such a field contains approx. 250 unit cells in one layer of the parallelogram channel array [1].) Patterns were recorded only from images of membranes shaped like collapsed cylindrical tubes, the morphological class of crystalline mitochondrial outer membranes which normally display the sharpest, highest resolution optical diffraction patterns. For each specimen, the best attainable pattern from each of 30–40 different membrane images was recorded. These patterns were scored blind, i.e., the scorer did not know to which specimens the different patterns corresponded. Membranes which showed at least three orders of sharp maxima (corresponding to a Fourier resolution of approx. 0.25 nm^{-1}) were scored as 'ordered'. Membranes showing only diffuse, low-order maxima or none at all were scored as 'disordered'.

Results

Ordering of channel protein induced by phospholipase A_2

The typical electron microscopic appearance of negatively stained mitochondrial outer membranes after overnight dialysis against low-salt buffer (specimen 1) is indicated in Fig. 1A. The membranes are predominantly large (up to 1.5 μm diam.), round vesicles with irregular, ruffled outlines. Inclusion of phospholipase A_2 during dialysis [1,6] results in the dramatic change in morphology demonstrated in Fig. 1B (specimen 2). The membranes are, on average, smaller (typically 0.4–0.8 μm diam.) and have smoother, unruffled outlines. Of special interest is the class of membranes in the phospholipase A_2-treated specimen which have periodic substructure (e.g., arrows and inset, Fig. 1B). The crystalline arrays in such membranes have been shown to contain the pore-forming 31 kDa polypeptide of the mitochondrial outer membrane [4]. In this particular preparation, 65% of the membranes in images of the phospholipase A_2-treated specimen gave optical diffraction patterns indicating crystalline substructure, compared with less than 1% in the untreated specimen.

Effects of phospholipases C and D

Unlike the case with phospholipase A_2, inclusion of either phospholipase C or phospholipase D in the membrane suspensions during dialysis (specimens 3 and 4) results in no obvious change in morphology relative to the control: the membranes are large and irregularly shaped with no evidence of ordered substructure (Fig. 1C and D). When these same specimens are subsequently treated with phospholipase A_2 (specimens 3′ and 4′), the membranes appear smaller with more regular, less ruffled outlines (Fig. 2A and B). However, few of the membranes in these specimens display the straight edges or obvious periodic substructure associated with membranes treated with phospholipase A_2 alone. Optical diffraction from these images confirm that long-range

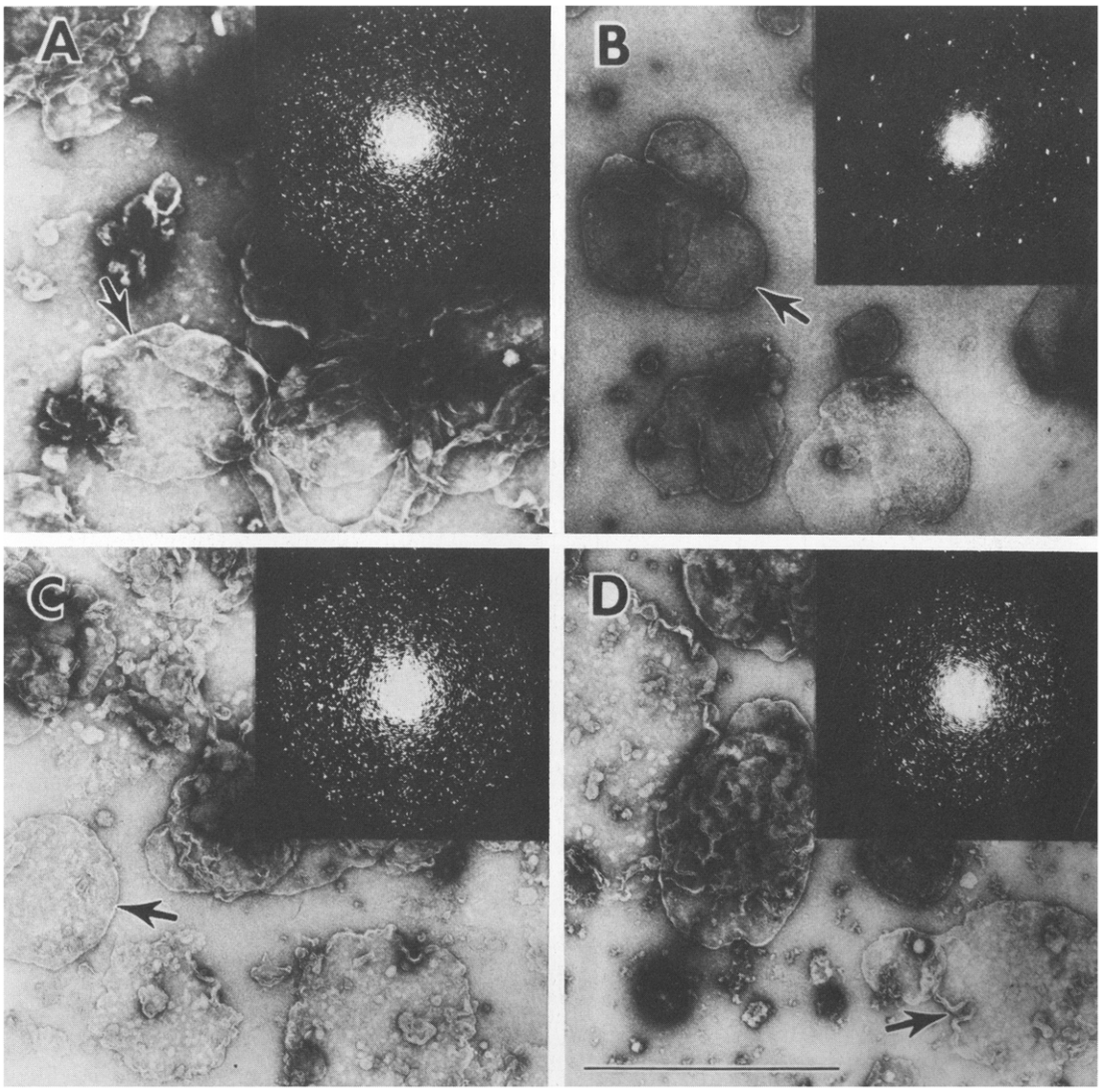

Fig. 1. Effects of phospholipases on structure of mitochondrial outer membranes. (A–D) Negative-stain electron micrographs of typical fields of membranes following overnight dialysis in presence of (A) no enzyme, (B) phospholipase A_2, (C) phospholipase C, and (D) phospholipase D. Arrows point to the membranes from which the optical diffraction patterns in the insets were recorded. Note that only the diffraction patterns from membranes in (B) display discrete reflections characteristic of the periodic channel arrays. Bar in (D) is 1 μm.

order in these membranes is, at best, very weak (inset, Fig. 2B).

The effects of treating the membranes first with phospholipase A_2 and subsequently with phospholipases C or D (specimens 2′a and 2′b) are illustrated in Fig. 2C and D. The general size and outline of the membranes are like those of membranes treated with phospholipase A_2 alone (Fig. 1B). In particular, there are numerous, straight-edged membranes bearing channel arrays. However, optical diffraction from many of the crystalline membranes in these fields (insets, Figs. 2C

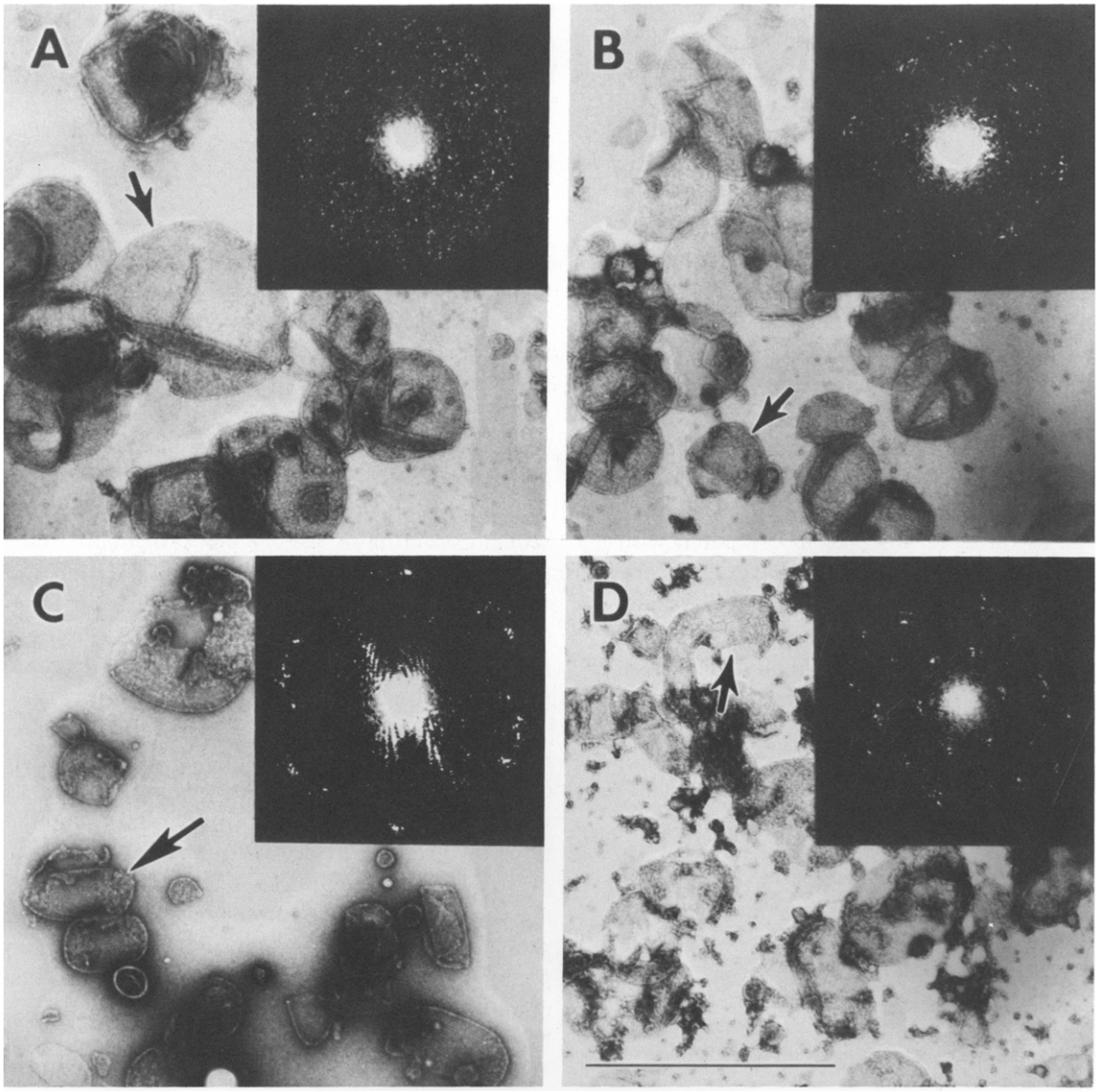

Fig. 2. Effects of phospholipases C and D on phospholipase A_2-induced ordering of mitochondrial outer membrane channels. Electron micrographs and optical diffraction patterns of typical membranes in specimens treated successively with (A) phospholipases C and A_2, (B) phospholipases D and A_2, (C) phospholipases A_2 and C, and (D) phospholipases A_2 and D. Note that the diffraction pattern of the membrane in (C) is that of the less common, rectangular type of channel array [6]. Bar in (D) is 1 μm.

and 2D) display maxima that are blurred or streaked, indicating decreased long-range order in the arrays *.

The time-course of the disordering effects of phospholipases C and D on periodic arrays of channel protein was followed in separate experiments. As before, membranes which had been pretreated with phospholipase A_2 were incubated

* The increased lattice disorder in crystalline membranes of specimen 2'b is not simply due to aggregation of the membranes with the small globular particles in these fields. Optical diffraction patterns were recorded only from areas on membranes with no apparent surface irregularities, such as particles or folds. The 15–20 nm particles observed in phospholipase D-treated specimens are oligomeric complexes of the enzyme [12], which appear to have a high affinity for the phospholipase A_2-treated membranes.

with either phospholipase C or D during continuous dialysis against buffer 1. However, in the new experiments, the reactions were run at room temperature and the membrane concentration was increased (from approx. 2 to 250 μg protein/ml) so that aliquots could be used directly for negative-stain specimen preparation. To quantitate disordering effects on the membrane arrays, optical diffraction patterns were recorded from images of array-bearing membranes under standard conditions and scored blind for long-range order, as described in Materials and Methods. The results of this experiment are summarized in Fig. 3. There is a slow decrease in the degree of ordering in the periodic arrays in outer mitochondrial membranes when they are left at room temperature for many hours. Only 75% of the tube-shaped membranes are scored as 'ordered' after 20 h, compared with 94% after 0.5 h. However, the decrease in the numbers of 'ordered' membrane arrays is much more rapid in the presence of either phospholipase C or D. In the case of phospholipase C, the fraction of well-ordered crystalline membranes drops rapidly within the first 0.5 h, after which the rate of decrease approximately parallels that of the control. With the phospholipase D-treated specimen, the decrease in numbers of ordered membranes is more gradual initially, but the fraction of crystalline membranes at the last time point measured (20 h) is the lowest of the three specimens.

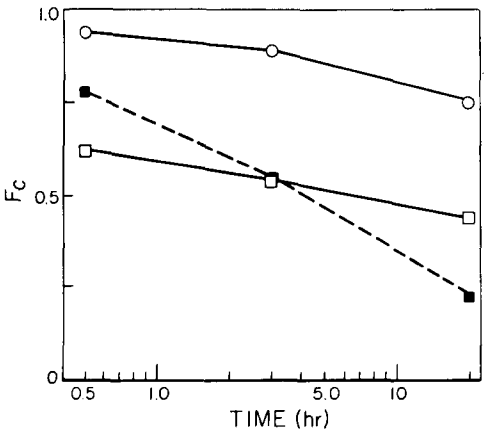

Fig. 3. Decrease in number of well-ordered channel arrays during treatment of mitochondrial outer membranes with phospholipases C and D. Phospholipase A_2-pretreated membranes incubated with either no additional enzyme (○), phospholipase C (□) or phospholipase D (■). Fraction of membranes bearing well-ordered crystalline channel arrays (F_c) was determined by optical diffraction from electron micrographs, as explained in text. Note that the 'time' scale is logarithmic.

Discussion

The basic conclusion to be drawn from the experiments described above is that phospholipases C and D, unlike phospholipase A_2, do not induce ordering of the channel protein in the outer membranes of *Neurospora* mitochondria. That these enzymes are altering the phospholipid composition of the membranes under the conditions employed in these experiments is indicated by two observations. First, pretreatment of the membranes with phospholipases C and D inhibits subsequent ordering of the channel proteins by phospholipase A_2 (Fig. 2A,B). Second, treatment of the membranes with phospholipases C and D under our reaction conditions causes disordering of pre-existing periodic arrays of the channel protein (Figs. 2C,D and 3).

These studies into the effects of different phospholipases on the organization of the mitochondrial outer membrane have been initiated with the goal of understanding the protein-lipid interactions involved in the crystallization of the channel protein in this membrane. Clearly, these studies are incomplete until a thorough analysis (in progress) of the changes in lipid composition accompanying these ultrastructural transitions has been completed. However, even in the absence of these biochemical data, certain tentative conclusions about the interactions between the channel protein and lipids can be drawn solely from the electron microscopic data. In particular, the finding that both phospholipases C and D inhibit channel crystallization and disorder existing crystalline arrays suggests that maintenance of long-range order in the arrays requires the presence of zwitterionic phospholipids. Maps of the mass density of the channel arrays (obtained from electron microscopic images of unstained arrays embedded in vitreous ice [10]) indicate that over 40% of the surface area of the arrays is occupied by lipid. That phospholipids are an important component of the arrays is indicated by the tendency of the lipid domains to be stained by uranyl salts [13].

The present results suggest that the zwitterions phosphatidylcholine and/or phosphatidylethanolamine (which constitute two-thirds of the phospholipids in the native *Neurospora* mitochondrial outer membranes [14]) will be found to be important components of the channel arrays induced by phospholipase A_2.

The amino-acid composition of the pore-forming polypeptide in the mitochondrial outer membrane is known for both fungi and animals [2,15], and the amino-acid sequence has been inferred from the cloned gene from yeast [16,17]. Between one-fourth and one-third of the amino-acid residues in these proteins carry a net charge at physiological pH. It may be that electrostatic interactions between charged amino acids and the polar head groups of zwitterionic phospholipids are involved in stabilizing the periodic arrays of channels which form in the outer membranes of *Neurospora* mitochondria.

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