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FUNCTIONAL AND STRUCTURAL CHANGES INDUCED BY PHOSPHOLIPASE C IN INTACT MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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SUMMARY

(1) Phospholipase C from *Bacillus cereus* causes uncoupling both in intact mitochondria and sonic particles. The uncoupling effect in intact mitochondria occurs parallel to the loss of osmotic properties, while in particles there is an inhibition of membrane bound enzymes. The kinetics of phospholipid hydrolysis and uncoupling are preceeded by a lag phase in mitochondria but not in particles.

(2) The extent of phospholipid hydrolysis by acid-base titration is 55 % and 43 % in mitochondria and particles, respectively. Chemical analysis gives an extent of hydrolysis of 38 % and 30 %, respectively. The degree of hydrolysis of phosphatidylcholine and phosphatidylethanolamine is the same in mitochondria and particles. Cardiolipin is not hydrolyzed.

(3) Fatty acids with spin label near the phospholipid polar heads indicate the formation of fluid hydrophobic regions in mitochondria. Also the pyrene fluorescence indicates an increased fluidity in mitochondria. In addition the phospholipase C attack is accompanied both in mitochondria and particles by an apparent increased immobilization of the spin label.

(4) The data are discussed in terms of the structural differences between mitochondria and particles and of a dual role of phospholipids in controlling enzymatic activities and osmotic properties.

INTRODUCTION

Phospholipases have been extensively used to study lipid-protein interactions and phospholipid organization in biological membranes. Verkleij et al. [1], observed an asymmetric distribution of phospholipids in the red cell membrane through the use of various phospholipases. Glaser et al. [2], concluded that protein conformation was independent of phospholipid polar heads because of the absence of changes in

Abbreviations: FCCP, carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone; I_E, fluorescence intensity of pyrene excimer; I_M, fluorescence intensity of pyrene monomer.

protein circular dichroism after extensive phospholipase C attack. In the case of mitochondria, Burstein et al. [3, 4] reported a loss of active transport of Ca^{2+} and of phosphorylation after treatment of mitochondria with phospholipase C. The change was attributed to membrane fragmentation and formation of submitochondrial particles. Structural changes have also been observed in phospholipase C treated kidney mitochondria [5].

In the present paper the functional and structural properties of rat liver mitochondria and submitochondrial particles were studied during treatment with phospholipase C. Although phospholipase C has been found to cause uncoupling, both in mitochondria and submitochondrial particles, the effect is attibuted to loss of osmotic properties in the former and to inhibition of membrane bound enzymes in the latter case, rather than to membrane fragmentation.

Two other differences between intact mitochondria and submitochondrial particles appear during phospholipase C attack, namely in the kinetics of the phospholipid hydrolysis, and in the response of structural probes such as spin label fatty acids and pyrene fluorescence. These differences, appearing during sonication, may be attributed either to inversion of the membrane or to changes in protein-lipid interactions. The similarity of the phospholipids hydrolyzed in the two cases favours a perturbation of the membrane structure.

EXPERIMENTAL

Rat liver mitochondria were prepared in 0.25 M sucrose, 5 mM Tris · Cl pH 7.4, 1 mM EDTA and washed twice [6]. Beef heart mitochondria and submitochondrial particles, from both rat liver and beef heart, were prepared as described previously [7, 8]. Liposomes, from soya beans, were prepared by sonicating ten times for two min, in 0.1 M KCl, 0.01 M Tris · Cl, pH 7.4, 2 mM EDTA, at 0 °C under nitrogen. The suspension was centrifuged at $40\,000 \times g$ for ten min and the supernatant was used either directly or after fractionation on a Sepharose 4B column. The NADH and pyrene fluorescence were measured either with an Eppendorf fluorometer or with a fluorescence spectrophotometer Hitachi Perkin Elmer, Model MPF 2 A. Oxygen uptake was measured with a Clark Electrode suitably connected with a recorder. When necessary, samples were taken from the cuvette and assayed for ATP synthesis. The movements of H^+ and K^+ were measured either directly on the basis of electrometric measurements with the H^+ and K^+ electrodes, or indirectly on the basis of absorbance changes. In the latter case the absorbance of the mitochondrial suspension was recorded continuously either with an Eppendorf Photometer at 546 nm or with a Perkin Elmer spectrophotometer Model 124 at 600 nm. The degree of energy coupling of mitochondria was measured in three ways, namely: (a) respiratory control, (b) endogenous NADH fluorescence and (c) energy linked safranine response. In this latter case the experimental conditions were as indicated by Colonna et al. [9] and the absorbancy changes were followed in a dual wavelength spectrophotometer. The degree of energy coupling in submitochondrial particles was followed through the energy linked acridine orange response, driven either by ATP or by succinate, as indicated by Dell'Antone et al. [10]. ATP synthesis was determined as described previously [11].

The kinetics of the phospholipid hydrolysis was determined by following in a

dual wavelength spectrophotometer the absorbancy changes of the pH indicator phenol red at the wavelengths 562–623 nm. In each experiment the absorbancy change was titrated against a standard solution of NaOH. The validity of the phenol red procedure was checked by measuring in the supernatant, after centrifugation, the liberation of P_i. The agreement between the two assays was excellent. All experiments described in the present paper were carried out with a highly purified preparation of phospholipase C from *Bacillus cereus* kindly provided to us by Van Deenen, L. L. M.

Phospholipid analyses

The phospholipase C from *B. cereus* was added to a membrane suspension containing 1 mg protein/ml and incubated at room temperature. The digestion was stopped by addition of organic solvents.

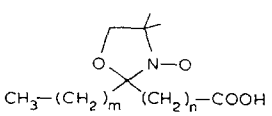
The membranes were treated with chloroform/methanol (2 : 1) for extraction of the phospholipids according to the procedure of Folch et al. [12]. The extracts were examined by thin layer chromatography with silica gel G plates (0.25 mm thickness) using chloroform/methanol/H_2O (65 : 25 : 4 by vol.) as developing solvent.

The spots were visualized by treatment with iodine vapor. The following reagents were also used: (a) the Dragendorff reagent in the case of phosphatidylcholine; (b) ninhydrin in the case of phosphatidylethanolamine and phosphatidylserine. Diphosphatidylglycerol was identified by reference with a standard of a pure sample.

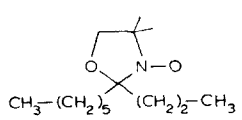
The phospholipid content of each spot was determined in two ways: (a) by measuring the phosphorus content of the scraped material after digestion with 70 % $HClO_4$ at 180 °C [13] and (b) by direct reading at 240 nm the reflectance of the iodine treated plates through the thin-layer chromatography accessory for the MPF fluorescence spectrophotometer. Protein was determined by the method of Lowry et al. [14] or by the biuret method [15].

ESR measurement

The structural changes caused by phospholipase C on mitochondrial and submitochondrial particles membranes have been followed by using spin label fatty acids possessing the general formula:



Two types of fatty acids were used: (a) $m = 12$, $n = 3$ and $m = 1$, $n = 14$. Both were commercial products, provided by SIVAR and were used directly. Another spin label compound was also used, posesssing the formula:



This was kindly provided by Drs. Seelig and Franklin of the Biozentrum, Basel.

The spin label fatty acids, when situated in a biological membrane, undergo an anisotropic motion according to an axis parallel to the phospholipid hydrocarbon

chains. Oscillations during this motion depend on the fluidity of the environment and may be expressed by an order parameter, S, introduced by Seelig [16] provided by:

$$S = 1/3\ (3\cos^2 v - 1) \tag{1}$$

where v is the angle between the axis perpendicular to the phospholipid surface and the axis of the N-O group. The order parameter may experimentally be calculated by Eqn. 2.

$$S = \frac{T_{||} - T_{\perp}}{T_{zz} - 0.5\,(T_{xx} + T_{yy})} \cdot \frac{a}{a'} \tag{2}$$

where the ratio a/a' is a correction introduced by Hubbell and McConnell [17] for the polarity of the medium. The following values were used for $T_{xx} = 31.91$; $T_{yy} = 5.83$ and $T_{zz} = 6.31$. $T_{\perp}$ and $T_{||}$ were determined from the ESR spectra [16, 17].

RESULTS

Phospholipase C attack in mitochondria

In a first series of experiments the effect of phospholipase was followed with mitochondria incubated for various periods of time with phospholipase C and then supplemented with EDTA [3]. This procedure yielded erratic results presumably due to incomplete inhibition by EDTA. The phospholipase C effect on the mitochondrial

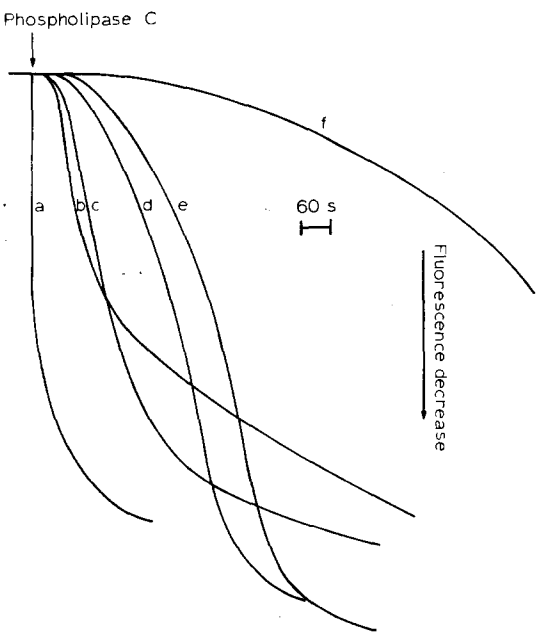


Fig. 1. Effect of various phospholipase C concentrations on NADH fluorescence. The medium contained 0.25 M sucrose, 10 mM Tris · Cl pH 7.4, 1 mM β hydroxybutyrate, and 2.5 mg mitochondrial protein. Amounts of phospholipase C were: (b) 0.8, (c) 0.48, (d) 0.16, (e) 0.08, (f) 0.024 I.U. of phospholipase C. In (a) was added 2 μM FCCP. Final volume 2.5 ml. Fluorescence was measured in an Eppendorf fluorimeter.

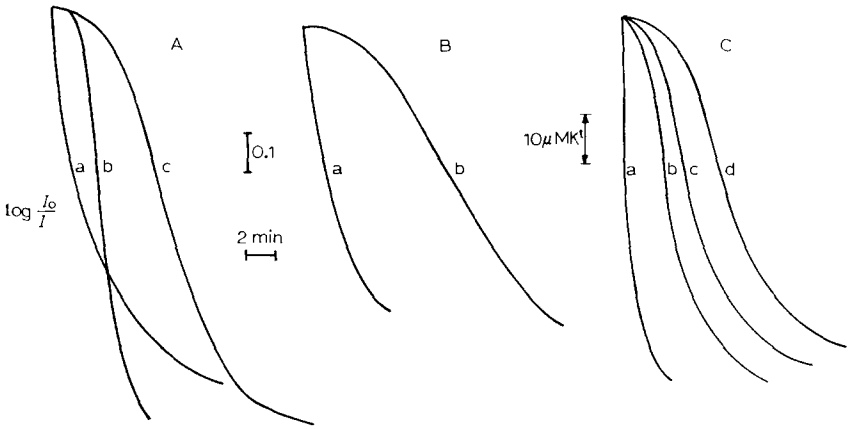


Fig. 2. Effect of phospholipase C on permeability to K^+ and H^+. (A) The medium contained 50 mM K_2HPO_4, 1 mM EDTA pH 7.5, 1 μM rotenone and 2 mg protein. When indicated (a) 2 μg nigericin or (b) 1.6 or (c) 0.32 I.U. of phospholipase C were added. (B) The medium contained 30 mM KI, 10 mM Tris · Cl, 1 mM EDTA pH 7.4, 1 μM rotenone and 2 mg protein. When indicated (a) 2 μg valinomycin or (b) 0.3 I.U. of phospholipase C were added. (C) The medium contained 0.25 M sucrose, 10 mM Tris · Cl pH 7.4 and 2 mg protein. When indicated (a) 2 μg valinomycin 1 μM FCCP, 100 μM KCl (b) 1.6 (c) 0.8 or (d) 0.32 I.U. of phospholipase C were added. Final volume 2 ml.

function was then recorded continuously. Fig. 1 shows that the endogenous NADH fluorescence of intact liver mitochondria was decreased at rates increasingly parallel to the amount of phospholipase C. Even at the highest phospholipase C concentrations the decrease of NADH fluorescence initiated after a lag phase lasting about 60 s. The lag phase was longer at the lower phospholipase C concentrations. For comparison the effect of FCCP is shown in Fig. 1. The decrease on NADH fluorescence was accompanied by inhibition of other mitochondrial activities such as release of respiratory control, decrease of phosphorylation and inhibition of succinate oxidation. Fig. 2 shows the effect of phospholipase C on K^+ and H^+ permeability. In Fig. 2 A mitochondria were incubated in 50 mM $K_2H\,PO_4$ pH 7.5. In this medium the penetration of P_i is limited by the rate of H^+/K^+ exchange. Addition of nigericin, which catalyzes a rapid H^+/K^+ exchange, caused swelling [18]. Addition of phospholipase C also caused swelling and the effect was proportional to the concentration of phospholipase. As in the case of the decrease of NADH fluorescence, the initiation of the H^+/K^+ exchange was preceded by a lag phase. In Fig. 2 B the mitochondria were incubated in 30 mM KI. In this medium the penetration of KI is dependent on the rate of K^+ permeation, I^- being a permeant anion. Addition of valinomycin caused swelling [18]. Similarly, addition of phospholipase C caused swelling due to high K^+ permeation. In Fig. 2 C, mitochondria were incubated in 0.25 M sucrose. Addition of phospholipase C caused a loss of K^+ and an uptake of H^+. The permeability of the mitochondrial membrane to other species was also increased, for example to inorganic anions and to sucrose. It may be concluded that treatment with phospholipase C causes a loss of osmotic properties of the mitochondrial membrane. These experiments may explain why addition of phospholipase C to intact mitochondria, results in inhibition of succinate oxidation and in uncoupling. The respiratory inhibition is due

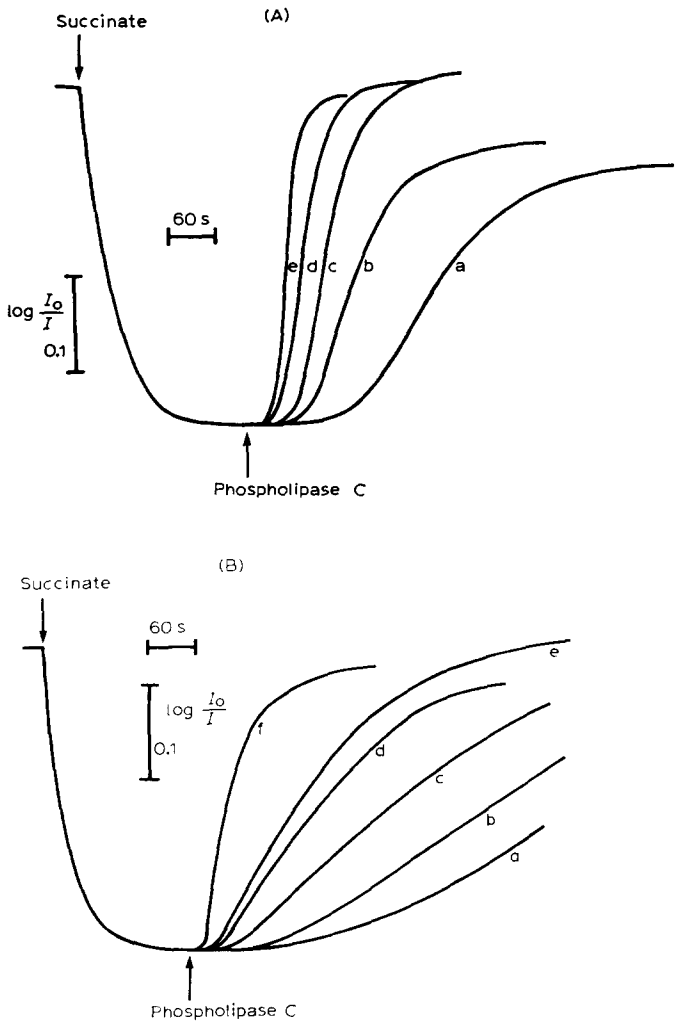


Fig. 3. A and B. Effect of various phospholipase C concentrations on safranine response in intact mitochondria and ghosts. The medium contained 0.25 M sucrose, 10 mM LiCl, 5 mM $MgCl_2$, 10 mM Hepes pH 7.4, 40 μM safranine and 2 mg protein. The reaction was initiated with 5 mM succinate. After reaching equilibrium the following amounts of phospholipase C were added in A: (a) 0.16, (b) 0.32, (c) 0.48, (d) 0.8 and (e) 1.6 and in B: (a) 0.16, (b) 0.32, (c) 0.48, (d) 0.8, (e) 1.6, (f) 2.4. All values are expressed in I.U. In A: intact mitochondria; in B: mitochondrial ghosts obtained through swelling in distilled water followed by osmotic shrinkage. Final volume 2 ml.

to loss of matrix K^+ and release of intramitochondrial metabolite anions. The uncoupling is due to loss of osmotic properties.

The lag phase during phospholipase C attack may be interpreted as due to the presence of the mitochondrial outer membrane which restricts access of the enzyme to the inner membrane. Fig. 3 shows that this is not the case. Addition of phospholipase C to intact mitochondria inhibited the energy linked safranine response,

proportionally to the amount of phospholipase. The effect was preceeded by a lag phase. Addition of phospholipase C to mitochondrial ghosts, which are depleted of the outer membrane, caused a reversal of the safranine response, also preceeded by a lag phase.

Experiments similar to those reported in Figs. 1–3 were also obtained with beef heart mitochondria. This excludes an involvement of endogenous phospholipase A_2 which is absent in beef heart mitochondria.

Phospholipase C attack in submitochondrial particles

Fig. 4 shows that addition of phospholipase C to submitochondrial particles inhibited the energy linked acridine orange response. The kinetics of the phospholipase C effect was different in the case of the ATP and the succinate induced acridine orange response. In the former case there was an initial rapid phase of inhibition of acridine orange response followed by a slower phase. In the latter case the inhibition was slower and partial. The inhibitory effect of phospholipase C in submitochondrial particles was not preceeded by the lag phase observed with mitochondria.

Addition of phospholipase C caused also an inhibition of the oxidation of NADH and succinate but not of ascorbate/TMPD. This type of inhibition may be interpreted as due to attack of the respiratory chain in the cytochrome *b* - *c* region.

Phospholipid hydrolysis

Fig. 5 A shows the correlation between uncoupling, loss of osmotic properties and phospholipid hydrolysis. It is seen that the kinetics of swelling and of safranine release were identical indicating that uncoupling proceeded parallel to the increase of ion permeation. The lag phase in uncoupling and swelling was paralleled by a lag phase in phospholipid hydrolysis. It may be calculated that the uncoupling and swelling initiated after about 8 % of the phospholipid was hydrolyzed. Fig. 5 A shows also that the extent of hydrolysis was about 58 % of the total phospholipids. 100 %

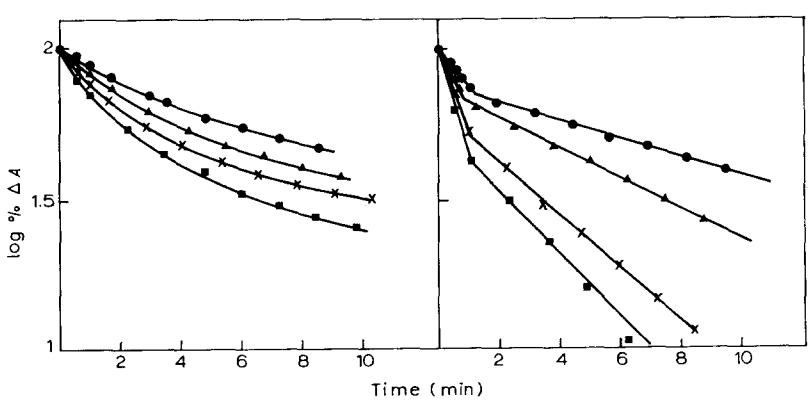


Fig. 4. Effect of phospholipase C on acridine orange response in submitochondrial particles.The medium contained 0.1 M KCl, 10 mM Tris · Cl pH 7.5, 5 μM AO, 5 mM $MgCl_2$. In the left part energy was supplied by 1 mM succinate, in the right part by 1 mM ATP. After reaching equilibrium the following amounts of phospholipase C were added ● 0.8; ▲ 1.6; × 3.2; ■ 4.8. All values are expressed in I.U.

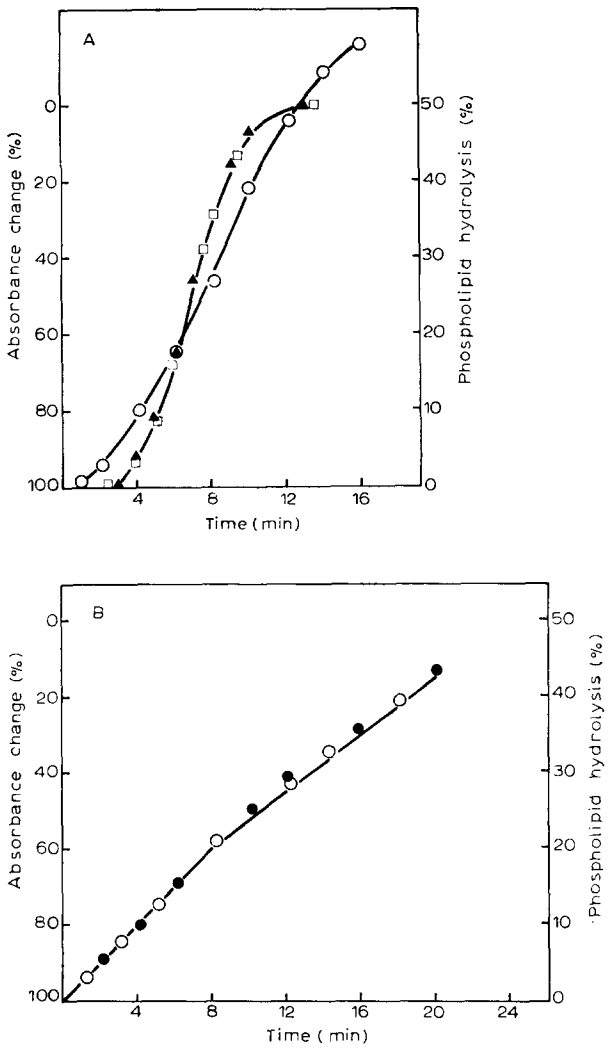


Fig. 5. A and B. Correlation between phospholipid hydrolysis, uncoupling and swelling. In A the medium contained 2 mg of rat liver mitochondria: ○, 0.1 M KCl, 2.5 mM Tris · Cl pH 7.5, 5 mM $MgCl_2$ and 50 μM phenol red; ▲, experimental conditions as in Fig. 3 to follow safranine response; □, experimental conditions as in Fig. 2A to follow penetration of K_2HPO_4. In B the medium contained 2 mg of submitochondrial particles: ○, 0.1 M KCl, 2.5 mM Tris · Cl pH 7.5, 5 mM $MgCl_2$, 50 μM phenol red; ●, experimental conditions as in Fig. 4 to follow acridine orange response driven by ATP. In all cases the reactions was initiated by 0.32 I.U. of phospholipase C.

uncoupling occurred at about 50 % phospholipid hydrolysis. Fig. 5 B shows the kinetics of uncoupling and phospholipid hydrolysis in submitochondrial particles. Addition of phospholipase C caused an initial rapid hydrolysis and uncoupling without lag phase. There was a good correspondence between the two kinetics. Complete uncoupling occurred at about 43 % hydrolysis. The extent of hydrolysis was about 10 % lower than in mitochondria.

TABLE I

HYDROLYSIS OF PHOSPHOLIPIDS BY PHOSPHOLIPASE C

The chemical determinations of the hydrolyzed phospholipids were carried out as described in the Methods. The data are reported as % hydrolysis in respect to the total phospholipids or to the single fractions.

	Extent of phospholipid hydrolysis (%)		
	Total phospholipids	Phosphatidyl-choline	Phosphatidyl-ethanolamine
Rat liver mitochondria	38.4±2.5	60.1±4.5	44.6±5.0
Beef heart mitochondria	36.5±2.0	58.0±2.0	51.5±2.5
Rat liver mitochondrial ghosts	36.5±3.5	59.0±5.0	59.0±4.5
Beef heart submitochondrial particles	30.3±4.5	48.0±5.3	40.0±5.2

The rate of phospholipid hydrolysis was also studied in mitochondria and submitochondrial particles at various concentrations of membrane protein. The membrane concentration required for half maximal rate of hydrolysis was one order of magnitude lower in the case of submitochondrial particles in respect to the intact mitochondria. On the other hand the maximal rate of hydrolysis was considerably higher in the case of mitochondria in respect to submitochondrial particles. The shift from the mitochondria to the submitochondrial particles type of behaviour occurred proportionally to the sonication time. Whether this shift is related to increase of membrane surface or to perturbation of membrane structure is not known at present. That the second alternative may be correct is suggested by the observation that a small amount of nonionic detergents caused a marked acceleration of the rate of phospholipid hydrolysis in liposomes and submitochondrial particles but not in mitochondria.

Table I shows a summary of the chemical analysis of mitochondrial phospholipid after phospholipase C hydrolysis as carried out under the same conditions as for the kinetic experiments of Fig. 5. It is seen that the extent of hydrolysis was about 40 % in the case of rat liver and beef heart mitochondria and about 30 % in the case of submitochondrial particles. These figures are about 10 % lower in respect to the extent of hydrolysis measured through the pH titrations (compare with Fig. 5). This may be due to incomplete extraction of the hydrolyzed phospholipids during the chemical determinations. Table I shows also that no significant differences were found in the amounts of phosphatidylethanolamine and of phosphatidylcholine hydrolyzed in mitochondria and submitochondrial particles. Cardiolipin was not hydrolyzed either in mitochondria or submitochondrial particles. In other experiments the time course of the hydrolysis of the main phospholipid components was measured. However the % of hydrolysis of each component were about the same after various times of incubation.

Structural effects

In Fig. 6 is shown the ESR spectrum of the fatty acid spin label ($m = 12$, $n = 3$), incorporated into mitochondria. The ESR spectrum indicated a complete penetration of the spin label within the membrane with an order parameter $S = 0.67$.

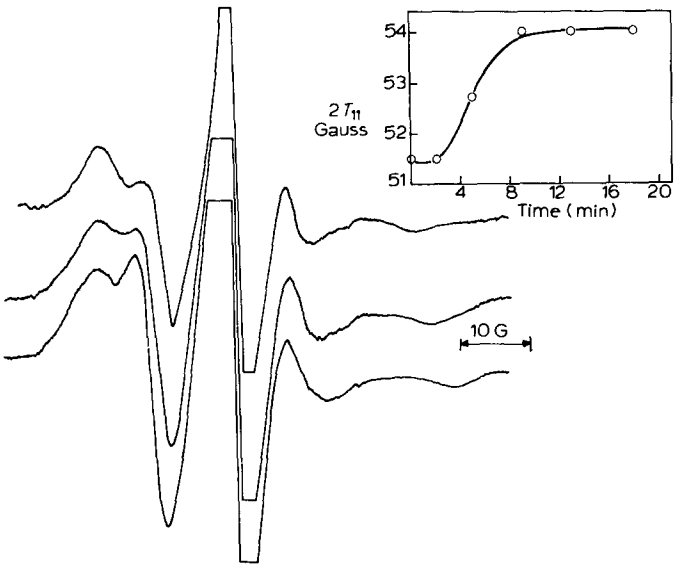


Fig. 6. Modifications of spin label response induced by phospholipase C in intact mitochondria. Experimental conditions were identical to those of Fig. 1. Amount of protein was 10 mg/ml. 1.6 I.U. of phospholipase C. Concentration of fatty acid spin label ($m = 12$, $n = 3$) 10^{-4} M. Each ESR spectrum was carried out at a scanning time of 25 gauss/min, time constant 0.3 s, modulation 1.25 gauss, temperature 22 °C. The insert shows a plot of 2 $T_{||}$ vs. time.

Addition of small amounts of phospholipase C caused no change in the ESR spectrum for about 2 min. After this period there was the gradual appearance of a new signal, so that the spectrum became the sum of two different spectra, one reflecting the degree of immobilization of the spin label, and another reflecting a fluid hydrophobic region. In order to determine the kinetics of the effect of phospholipase C on these two parts of the spectrum, the following procedure was used. As to the degree of immobilization of the spin label, the phospholipase C effect was related with the parameter $T_{||}$ which actually reflects the immobilization of the spin label. In the insert of Fig. 6 is shown a plot of 2 $T_{||}$ as a function of time. It is seen that phospholipase C caused an increase of $T_{||}$, i.e. increased immobilization, after a lag phase similar to that of Figs 1–3.

In order to follow the kinetics of the formation of the fluid region an additional parameter α has been introduced. This parameter is defined as the h_1/h_2 ratio, where h_1 and h_2 are measured as indicated in Fig. 7; h_2 expresses the signal of the immobilized spin label before the addition of phospholipase C and h_1 is the signal of the phospholipase C-induced fluid hydrophobic region. In the insert of Fig. 7 is shown a plot of α as a function of time. Again the increase of α, reflecting the formation of the fluid region, occurred after a lag phase.

In Fig. 8 are reported the ESR spectra obtained after incorporation of the fatty acid spin label into submitochondrial particles ($m = 12$, $n = 3$). The ESR spectrum indicated a complete penetration of the spin label within the membrane. The order parameter S, indicating the extent of spin label immobilization, was 0.69. Addition of phospholipase C caused a gradual increase of the order parameter S indicating an

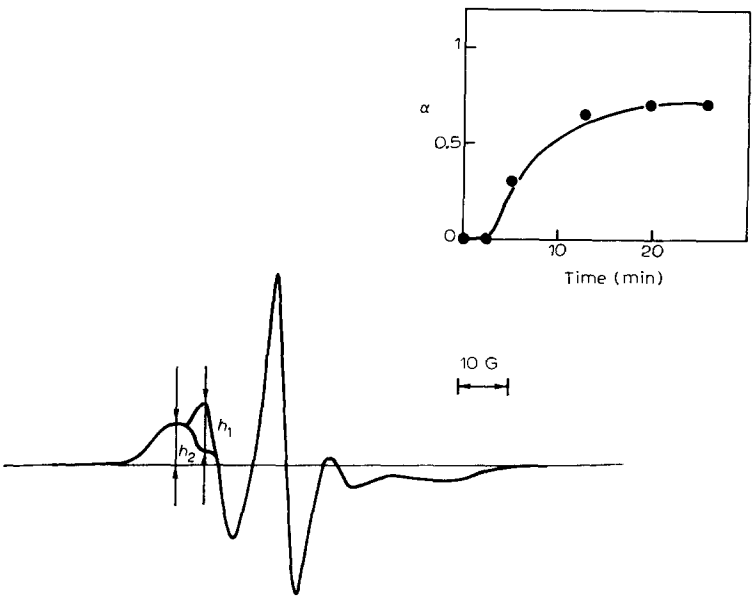


Fig. 7. Analysis of the ESR spectral changes. The experiment was that of Fig. 6, and the drawing indicates the procedure used to calculate the parameter $\alpha = h_1/h_2$. The insert shows a plot of α vs. time.

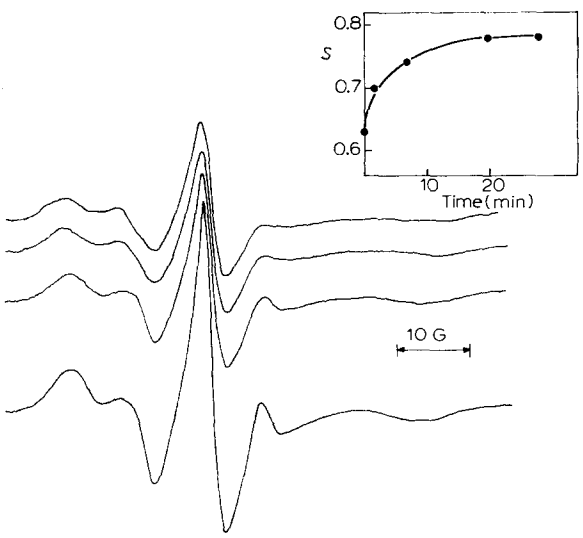


Fig. 8. ESR spectral changes in submitochondrial particles. The protein concentration was 3.5 mg. The ESR spectrum of the fatty acid spin label ($m = 12, n = 3$) was recorded under experimental conditions similar to those of Fig. 6. The amount of phospholipase C was 1.6 I.U. The insert shows a plot of S vs. time.

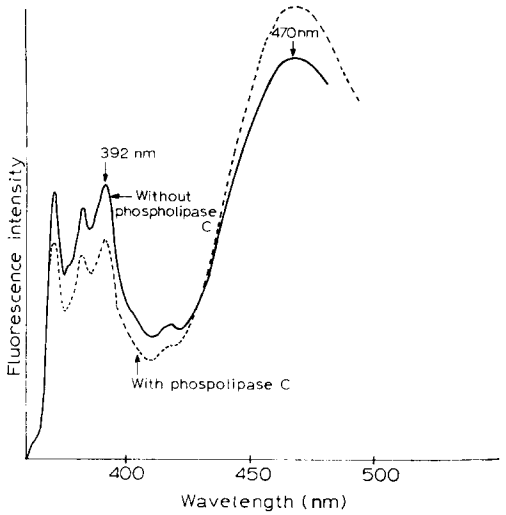


Fig. 9. Effect of phospholipase C on pyrene response in mitochondria. The medium contained 0.25M sucrose, 10 mM Tris · Cl pH 7.4, 20 μM pyrene and 2 mg protein. Final volume 2 ml. Excitation 320 nm. The dashed line shows the spectrum of pyrene after treatment of mitochondria with phospholipase C.

increasing rigidity of the membrane in a region close to the phospholipid polar heads. The kinetics of the increase of the order parameter S was similar to that of the abolition of the succinate induced acridine orange response (Fig. 4).

The fatty acid spin label ($m = 1$, $n = 14$) was used in order to ascertain whether there was an effect of phospholipase C also on the fluidity of the core of the phospholipid bilayers. The ESR spectra were recorded after addition of the fatty acid spin label ($m = 1, n = 14$). The shape of the spectrum does not permit the determination of $2\,T_{||}$ and therefore of the order parameter S. It is however possible to determine the rotational parameter τ introduced by Kivelson [20] which reflects mobility of the environment. The value of τ was $10 \cdot 10^{-10}$ s in mitochondria and was not appreciably modified by the phospholipase C. The ESR spectrum of the hydrocarbon spin label in the presence of mitochondria and submitochondrial particles was also recorded. The spectrum was similar to that of the fatty acid spin label ($m = 1, n = 14$), indicating also in this case a high fluidity of the environment. The rotational parameter τ was $7.9 \cdot 10^{-10}$ s and was not modified by the phospholipase C attack.

Soutar et al. [21] have reported that the degree of formation of the excimer of pyrene is a sensitive parameter of the microscopic viscosity of the hydrocarbon region in a phospholipid bilayer. The method is based on the viscosity dependence of the translational diffusion rate of the probe. Fig. 9 shows that treatment of mitochondria with phospholipase C results in a shift in the emission spectrum of pyrene which corresponds to an increased formation of excimer. The ratio of intensities of the excimer and the monomer, I_E/I_M, indicated a shift of about 20 % in favour of the excimer. No change of pyrene response was observed with submitochondrial particles.

DISCUSSION

At variance with the report of Burstein et al. [3, 4], in the present study the effect of phospholipase C was neither prevented nor restored by bovine serum albumin, it was not dependent on exogenous divalent cations and was not affected by the presence of chelating agents. The differences may be due to our using phospholipase C from *B. cereus* instead of phospholipase C from *Clostridium welchii*, and fresh rat liver mitochondria instead of stored beef heart mitochondria. The phospholipase C from *B. cereus* attacks both acidic and neutral phospholipids and exerts its effect in the presence of a negative zeta potential [22]. The phospholipase C from *Cl. perfringens* or *welchii*, attacks mainly neutral phospholipids and requires a positive zeta potential [23]. The accessibility of the phospholipids polar heads may also be different in fresh rat liver mitochondria and in stored beef heart mitochondria.

Because of the discrepancy between loss of phosphorylation and of active transport, Burstein et al. [3], proposed that the loss of Ca^{2+} uptake was not due to uncoupling but rather to an inversion of polarity of the membrane induced by phospholipase C. In the present study phospholipase C causes uncoupling both in intact mitochondria and submitochondrial particles. In intact mitochondria uncoupling accompanies a loss of osmotic properties. Phospholipase C causes increased permeability to ions and hydrophilic solutes. There is a close correlation between increase of permeability and decrease of coupling. The respiratory inhibition following phospholipase C treatment may be due either to an effect at the level of membrane bound enzymes or to the loss of metabolite anions from the mitochondrial matrix. In the case of submitochondrial particles it is impossible to decide whether the uncoupling is due to an increased membrane permeability. First, the extent to which the submitochondrial particles membrane still possesses osmotic properties is controversial. Azzone and Massari [22] reported the sonication causes an increased permeability of the membrane to high molecular weight solutes. Second, the kinetics of uncoupling is not the same when energy is supplied by succinate or by ATP (Fig. 4) as it should be in case of an osmotic effect. The rapid abolition of the ATP induced energy linked response is in accord with an effect of phospholipase C on the ATPase complex [25]. That membrane bound enzymes are more susceptible to phospholipase C attack in submitochondrial particles, as compared to mitochondria, is also in accord with the more marked inhibition of NADH dehydrogenase in the former system.

The ESR and fluorescence measurements reported in the present study support a distinction between effects of phospholipid hydrolysis at the level of intact mitochondria and submitochondrial particles. In the case of mitochondria, the ESR data indicate the formation of regions possessing high hydrophobicity and fluidity. Such regions are localized near the polar heads of the phospholipids and may correspond to the formation of diglyceride micelles. This would explain the loss of osmotic properties. The increase of pyrene excimer also indicates the formation of highly fluid regions in mitochondria. On the other hand the ESR data indicate an increased rigidity of the membrane regions around the spin labels. The increased rigidity may be the structural expression for the inhibition of some membrane bound enzyme. It is significant that the spin labels localized in the hydrophobic core of the membrane indicate no change of membrane fluidity under conditions where there is a dramatic

change of the osmotic properties. This suggests that the permeability of the bilayer is controlled at the level of the phospholipids polar heads.

Burstein et al. [3] reported that treatment with phospholipase C caused release of about 50 % of the total phosphorus in mitochondria. In the present study: (a) the extent of phospholipid hydrolysis is higher in mitochondria than in submitochondrial particles, 58 % as compared to 43 % and (b) no variation in the nature of phospholipids hydrolyzed is detected between mitochondria and submitochondrial particles. This is unexpected. Biological membranes possess an asymmetric distribution of proteins and, in the case of the red cell membrane, an asymmetric distribution of phospholipids. Mitochondria and submitochondrial particles are thought of as possessing a membrane with opposite polarity [26, 27] in view of different exposition of marker proteins, such as cytochrome *c* and F_1, at the outer surface [28]. Two explanations may be conceived for the observation that the phospholipids hydrolyzed are about the same in intact mitochondria and submitochondrial particles: (a) that most phospholipids in mitochondria are not concerned with the membrane asymmetry or (b) that sonication leads partially to scrambling and partially to a mixture of membranes with both polarities.

The kinetics of phospholipid hydrolysis reveals striking differences between mitochondria and submitochondrial particles. First, a lag phase is present in intact mitochondria but not in submitochondrial particles. Second, the concentration of phospholipid for half maximal rate of hydrolysis is much higher for mitochondria than for submitochondrial particles. Furthermore: (a) the phospholipid concentration for half maximal rate of hydrolysis is in liposomes close to that of submitochondrial particles and not to that of the native membrane and (b) detergents at low concentrations enhance the rate of hydrolysis in submitochondrial particles and liposomes but not in intact mitochondria. The pyrene fluorescence also indicates the presence of regions with higher fluidity in the case of intact mitochondria in respect to submitochondrial particles. All these findings suggest an increased accessibility of the active site of the enzyme for the phospholipid polar heads of submitochondrial particles.

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