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Phospholipase C and the Physical States of Polar Head Groups of Lipids

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Summary. Activity of phospholipase C from *Clostridium perfringens* on liposomes made from *sn*-3-phosphatidylcholine, dimyristoyl (DMPC), dipalmitoyl (DPPC) or distearoyl (DSPC) was measured at various temperatures and was correlated with their gel/liquid-crystalline phase transitions (T_c: 23, 41.5, 52°C for DMPC, DPPC, DSPC, respectively). In all cases, the activity of phospholipase C was high in the gel phases of the substrates and was almost zero in their liquid-crystalline phases. Fluorescence depolarization measurements of N-dansyl-*sn*-3-phosphatidylethanolamine (DPE) and 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the liposomes showed that both the head group and the alkyl chains of the lipids were immobilized in the gel phases but were highly mobile in the liquid-crystalline phase. These results indicate that the rotational mobility of lipids (both of the head groups and the alkyl chains) was not a major factor in the phospholipase C reaction. It is inferred that some electrostatic and/or hydrophobic interactions might play important roles in regulation of the phospholipase C activity.

Key Words phospholipase C · liposomes · fluorescence study · local dielectric property · rotational freedom

Introduction

Phospholipase C is the enzyme that hydrolyzes the glycero-phosphate ester bond and cleaves off polar head groups from phospholipids at water-lipid interfaces. They were originally found as toxins in bacteria (Ikezawa et al., 1983; van den Bosch, 1982). Now it is well known that some of these enzymes play the first crucial role in agonist-stimulating signal transduction in various cells (Michell, 1975; Hokin, 1985). In bacteria and cells, the enzyme activity seems to be well regulated and therefore can play important roles in vivo. The regulating mechanism, however, is not well understood, and detailed knowledge about the function of this enzyme may be necessary.

Phospholipase C reacts with lipids at a water-lipid interface and hence is affected by the physical properties of the interface. Electrokinetic and structural properties were reported to have strong influence on the reaction (Bangham & Dawson, 1962; Dawson, 1968; Jain & Cordes, 1973; Dawson et al., 1976). The enzyme reacted with bilayers only when the surface zeta-potential was made positive by addition of calcium to the aqueous phase or by incorporation of water-insoluble cations into the bilayers (Bangham & Dawson, 1962; Dawson, 1968). On the other hand, the enzyme activity increased without adding calcium when the membrane contained *n*-alkanols (Jain & Cordes, 1973; Goldhammer, Jain & Cordes, 1975; Dawson et al., 1976). The apparent inconsistency in calcium requirement was resolved by considering that only calcium bound to the enzyme was required in the presence of *n*-alkanols. The electrokinetic effect of calcium was understood as an expansion of the monolayer and a resultant increase in rotational motion of lipids, a similar effect to that exerted by *n*-alkanols (Dawson et al., 1976). Thus, both investigators concluded that the rotational freedom of lipids was the most crucial factor in the reaction of phospholipase C. However, the use of *n*-alkanols or some other chemicals is an indirect means to study the relationship between the enzyme activity and the substrate mobility. Thus, the conclusions were somewhat ambiguous.

I investigated the rotational motion of lipids and the local dielectric properties at the water-lipid interface by fluorescence measurements. The rotational motion was studied by measuring fluorescence depolarization of N-dansyl-*sn*-3-phosphatidylethanolamine (DPE) and 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated in the lipid bilayers of liposomes made of synthetic phosphatidylcholine and soybean phosphatidylcholine. DPE is a lipid-like molecule and has a fluorescent chromophore, dansyl, attached covalently to the amino group of *sn*-3-phosphatidylethanolamine, and thus is a good probe for the polar head-group region of lipid bilayers (Kimura & Ikegami, 1985). DPH is a hydrophobic molecule and is a good probe for the hydrophobic region of lipid

bilayers (Kinosita et al., 1981). The local dielectric properties were studied by measuring fluorescence spectra of DPE as described by Kimura and Ikegami (1985). It will be shown that the rotational mobility of lipids may not be an important factor in the phospholipase C reaction but some electrostatic and/or hydrophobic interaction plays important roles in the reaction.

Abbreviations

soybean PC, *sn*-3-phosphatidylcholine purified from soybean; DMPC, *sn*-3-phosphatidylcholine, 1,2-dimyristoyl; DPPC, *sn*-3-phosphatidylcholine, 1,2-dipalmitoyl; DSPC, *sn*-3-phosphatidylcholine, 1,2-distearoyl; DPE, *sn*-3-phospho(N-dansyl)ethanolamine, 1,2-dipalmitoyl; DPH, 1,6-diphenyl-1,3,5-hexatriene; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; T_c, gel/liquid-crystalline phase transition temperature.

Materials and Methods

Materials

Phospholipase C purified chromatographically from *Clostridium perfringens* (Type XII) and L-α-phosphatidylcholine (*sn*-3-phosphatidylcholine (dimyristoyl, dipalmitoyl and distearoyl) were purchased from Sigma (St. Louis, MO). Soybean phosphatidylcholine was purified as follows: Crude extract was purchased from Nakarai Chemical (Kyoto, Japan). First, it was dissolved in 1 vol chloroform with a small amount of methanol, and then it was precipitated by adding 9 vol acetone. The precipitate was dried, dissolved in chloroform again and purified on a DEAE-cellulose column and then on a silicic column. Eluates were fraction collected and each fraction was checked by thin-layer chromatography (Silica gel 60, Merck, developing solvent: chloroform/methanol/water, 65 : 25 : 4 and chloroform/methanol/acetic acid/water, 25 : 15 : 4 : 2; detection : iodine vapour, Dittmer reagent and Dragendorff reagent, *see* Kates, 1972). N-dansyl-*sn*-3-phosphatidylethanolamine (DPE) was synthesized according to Waggoner and Stryer (1970) and purified on preparative thin-layer plates (2 mm thickness). All lipids were handled under a nitrogen atmosphere. All other reagents were analytical grades purchased from Wako Pure Chem. Indust. (Osaka, Japan) or Nakarai Chemical, Ltd. Concentrations of lipids and DPE were determined by the method of Rouser and Fleischer (1967).

Preparation of Liposomes for Phospholipase C Reaction

Lipids were dissolved in chloroform/methanol (2 : 1 vol/vol) and a portion was dried in nitrogen stream and further in vacuum 2–4 hr (24 hr did not show any difference). The dried lipids were suspended in 20 mM HEPES buffer (pH 7.0) or sometimes in water, and the suspension was sonicated with a microtip sonicator (Branson sonifier 200, 30% pulse duration) for about 15 min above the phase transition temperatures of the lipids. The suspension became clear at this stage. The suspension was centrifuged at 2000 × *g* for 10 min to remove titanium chip fragments and then filtered on Milex GV filter (0.22 μm, Millipore) to remove possible aggregated liposomes. The suspension (typically 4–5 mM) was adjusted to a final concentration (2.33 mM) in a final buffer (20 mM HEPES, pH 7.0, 1 mM $CaCl_2$, 130 mM NaCl). Then the suspensions in test tubes were further sonicated in a bath-type sonicator (Branson Model B-3). In order to ascertain the chemical composition of lipids, the suspended lipids were re-extracted into chloroform according to Bligh and Dyer (1959) and were checked by thin-layer chromatography; no degradation was detected after the above processes. Liposomal states at the end of the experiments were checked by monitoring the turbidity of the samples.

Preparation of Liposomes for Fluorescence Measurements

Liposomes were prepared essentially by the same method as described above except that the filtration step was omitted and the final concentrations were: lipid, 0.22 mM; DPE, 1–2 μM; DPH, 0.2–0.3 μM.

Assay of Phospholipase C Activity

The final suspension of lipids (0.5–2 ml) was incubated for more than 15 min in a water bath at various temperatures between 10 and 60°C. The suspension was mixed with 5–20 μl of phospholipase C (66 μg/ml). Every 30 sec after mixing, a 180 μl sample was taken and mixed rapidly with 400 μl chloroform/methanol (1 : 1 vol/vol) and was centrifuged at 2800 × *g* for 10 min at 4°C. A portion of the upper methanol-water (180 μl) was taken and the phosphate in it was assayed according to Rouser and Fleischer (1967). To check the accuracy of the above method, mixtures of water-soluble phosphates and phospholipids were treated as above and phosphate amounts in both methanol-water and chloroform phases were assayed. The difference between calculated values and measured values were less than 2% ($n = 50$). In all cases, the initial time courses of the reaction (up to 2 min in most cases) appeared to be linear (*see* Fig. 2 for example) at all temperatures scanned in a direction from low to high. To check the liposomal states and reversibility, phospholipase C activities at temperatures scanned in the opposite direction were measured after treating the sample in the same manner as above.

Fluorescence Measurements

Fluorescence depolarization and fluorescence spectra were measured with a fluorescence spectrophotometer (Hitachi MPF-4) equipped with a water-circulating temperature control system. The temperature in the cuvette was measured with a calibrated thermister. For the fluorescence depolarization measurements, DPE and DPH were excited with vertically polarized light at 340, 360 nm ($\Delta\lambda = 5$ nm). The fluorescence was measured at 500, 450 nm ($\Delta\lambda = 5$–6 nm) through a polarizer set (either vertically, I_v, or horizontally, I_h) in 90° configuration. Built-in filters were used to minimize stray light. The fluorescence anisotropy, r, was calculated as

$$r = \frac{I_v - s \cdot I_h}{I_v + 2s \cdot I_h}$$

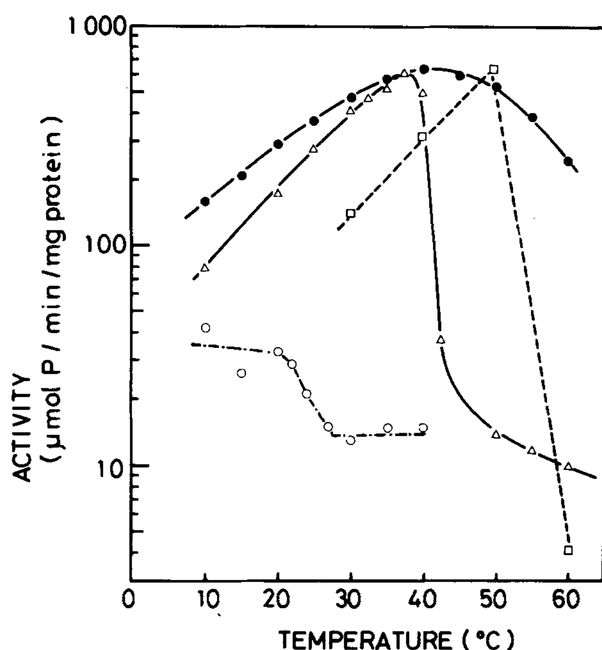

Fig. 1. Temperature dependence of phospholipase C activity. Lipids (●, soybean PC; ○, DMPC; △, DPPC; □, DSPC; final concentration 2.33 mM) were suspended and sonicated in buffer (20 mM HEPES, pH 7.0, 1 mM $CaCl_2$, 130 mM NaCl). Phospholipase C from *Clostridium perfringens* were added to the suspension and sampled 30, 60, 90 and 120 sec later. Sampled aliquots were immediately mixed with chloroform/methanol (1 : 1, vol/vol) and centrifuged. Phosphate amounts in the upper methanol/water phase were assayed

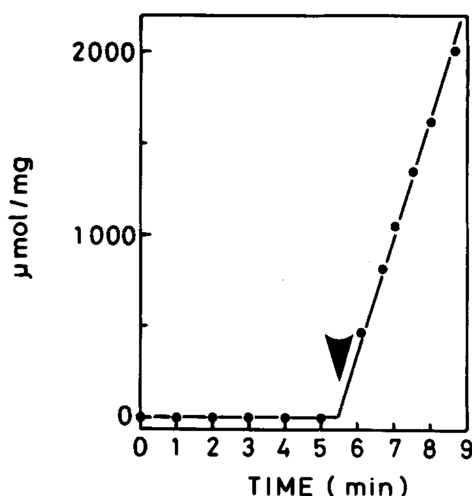

Fig. 2. Activity check of phospholipase C in DPPC liposomes. DPPC liposomes (2.33 mM) and phospholipase C (final 0.67 μg/ml) were mixed at 46°C. 330 sec later, soybean PC liposomes (thermally equilibrated at 46°C, final concentration 2.33 mM) were added. Assays were performed as in Fig. 1

where s is the ratio between sensitivities of the detection system to vertically and horizontally polarized lights and was determined as I'_v/I'_h for horizontally polarized excitation light. For fluorescence emission spectra, DPE was excited at 340 nm ($\Delta\lambda$ = 5 nm), and for excitation spectra, emission was measured at 500 nm ($\Delta\lambda$ = 5 nm). The relation between DPE emission spectra and dielectric constants was similar to that reported previously (Kimura & Ikegami, 1985) except that the emission peaks ranged from 480 to 520 nm due to the sensitivity difference of the system. Since spectra were assigned to a system-independent dielectric constant, further corrections for the sensitivities of the system were not necessary. Other details were the same as described by Kimura and Ikegami (1985).

Results

Temperature Dependence of Phospholipase C Activity

The phospholipase C activity for soybean PC ranged between 100 and 600 μmol min^{-1} mg^{-1} according to the reaction temperature (Fig. 1). The optimal temperature was 40–50°C, which depended on the particular preparation of phospholipase C. For DPPC, the activity showed a sharp reversible change at the phase transition temperature (T_c = 41.5°C). Below T_c, the activity for DPPC liposomes was not much different from the one for soybean PC liposomes. Above T_c, the activity for DPPC decreased almost to zero, though the activity for soybean PC remained high. An essentially similar activity change was observed for DMPC and DSPC liposomes at the phase transition temperatures 23 and 52°C, respectively. Though the absolute values and the optimal temperature of the activity varied with different preparations of phospholipase C, the essential features were consistently the same as shown in Fig. 1.

Activity Check of Phospholipase C in DPPC Liposomes

To check the possible denaturation and/or inactivation of phospholipase C in the suspension of DPPC liposomes above T_c, soybean PC was added to DPPC suspension 330 sec after mixing with phospholipase C (Fig. 2). The results clearly showed that phospholipase C remained highly active towards soybean PC but not to DPPC. Since lipid head groups have the same chemical structure in both phosphatidylcholines, the only possible factors that cause the difference are the physical states of the liposomes at their surfaces.

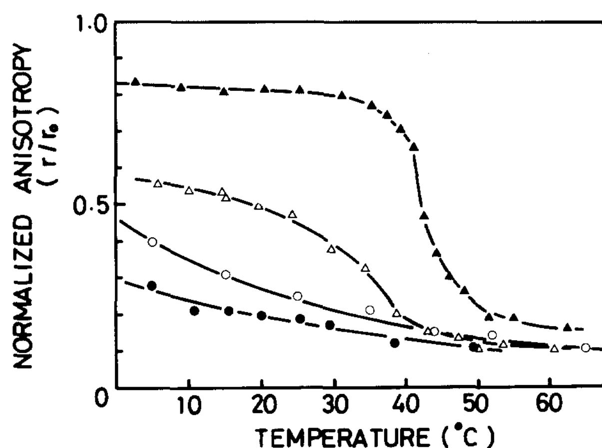

Fig. 3. Normalized fluorescence anisotropies of DPE (○, △) and DPH (●, ▲) in PC liposomes. DPE (1 μM) and DPH (0.3 μM) were embedded in PC liposomes (○, ●: soybean PC; △, ▲: DPPC; 0.22 mM). r_0 is 0.395 (DPH) or 0.280 (DPE). Buffers contain 20 mM HEPES, pH 7.0, 1 mM $CaCl_2$ and 130 mM NaCl

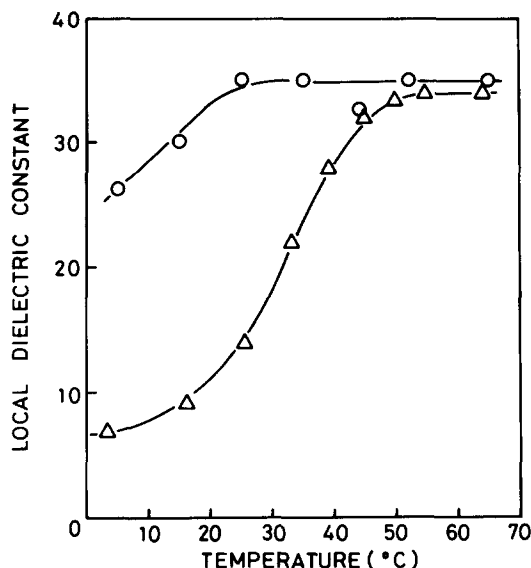

Fig. 4. Local dielectric constants around the polar region of PC liposomes. ○, △ are soybean PC and DPPC, respectively. Other conditions were the same as in Fig. 3

Dynamic Structures of the Polar Head Groups of Phosphatidylcholine Liposomes

Fluorescence anisotropies of DPE and DPH in soybean PC liposomes showed that the polar head groups and the alkyl chains of soybean PC were highly mobile at all temperatures but those of DPPC showed a marked change in mobility at the phase transition temperature (T_c) (Fig. 3). Below T_c, the polar head groups and the alkyl chains were immobilized (or less fluid from macroscopic view point). Above T_c, on the other hand, both were highly mobile. A difference between the polar head groups and the alkyl chains was that the alkyl chains showed a more prominent phase transition at 41.5°C in DPPC liposomes but that the polar head group of DPPC showed a less prominent change at lower temperatures. Essentially similar data were obtained for DSPC. All these data agree with the results of Kimura and Ikegami (1985) and also with those of previous works by other authors (Faucon & Lussan, 1973; Tessie, 1979).

Dielectric Constants around the Polar Head Groups in Phosphatidylcholine Liposomes

Dielectric constants measured by the DPE fluorescence were mostly higher than 30 for soybean PC, whereas they varied between 6 and 33 below T_c and stayed around 33 above T_c for DPPC (Fig. 4). Data of DSPC were similar to those of DPPC. Though the local dielectric constant in DPPC liposomes changed somehow parallel to the loss of activity of phospholipase C, their temperature profiles had distinct differences (Fig. 1 and 4). The local dielectric constant in DPPC liposomes changed gradually below the phase transition temperature and was constant above the transition temperature, whereas the activity changed sharply around the transition temperature. The temperature dependence of the dielectric constant was inversely proportional to the dependence of DPE fluorescence anisotropy in DPPC liposomes (Figs. 3 and 4; *see also* Kimura & Ikegami, 1985). The dansyl chromophore of DPE reflects the dielectric properties near the upper part of the polar head group. However, phospholipase C reacts with glycerophosphate ester bond located at the lower part of the head group. Therefore, the dynamic structure near the site of enzyme action may be more similar to that of the upper part of the alkyl chain region rather than to the upper part of the polar head group. The local dielectric properties reflect the dynamic structures of polar molecules (polar head group and local water). Therefore, the change of the local dielectric constant near the lower part of the head group of lipids may change sharply at the phase-transition temperature similar to changes in DPH anisotropy in Fig. 3.

Discussion

In the case of DPPC liposomes, the loss of activity of phospholipase C occurred abruptly at 40–42°C (Fig. 1). A similar drastic change at the same tem-

perature was observed by fluorescence depolarization study as shown in Fig. 3: DPH fluorescence anisotropy decreased drastically at the phase-transition temperature in DPPC liposomes. The DPE and DPH data indicate that both the polar head groups and the alkyl chains of DPPC are highly mobile above the phase-transition temperature. Therefore, the loss of activity above the transition temperature is inversely related to the increase in mobilities of both the lipid's head group and the alkyl chains. Results in Fig. 2 show that the enzyme's activity itself was maintained at a high level. Therefore, the apparent loss of the activity may be attributed to the physical conditions of the substrate. On the other hand, the observed dielectric properties for DPPC showed an increase to the maximal value at T_c in contrast to the decrease of the activity. Electrostatic and also hydrophobic interactions are inversely related to the dielectric properties. The observed loss of the activity above the phase-transition temperature might be explained by the lowered electrostatic and/or hydrophobic interaction due to the increase of the local dielectric constant at the polar head group region of the lipid bilayers.

In the case of soybean PC liposomes, however, the phospholipase C activity was high at all temperatures, probably because soybean PC have heterogeneous chain compositions. It is likely that fluorescent dyes probed mostly a fluid phase of the soybean PC, and hence the anisotropy was small and the local dielectric constant was high. Therefore, soybean PC liposomes were used for the activity check of the enzyme.

The present report shows that the rotational freedom of the head group and the alkyl chains of lipids was not a major factor in the phospholipase C reaction with DMPC, DPPC and DSPC liposomes. Some electrostatic and/or hydrophobic interaction around the reaction site probably play important roles.

The regulation of phospholipase C activity is a central question for understanding the physiological action of phospholipase C. A possible regulating mechanism through local electrostatic/hydrophobic interactions might open up new insights into the phospholipase C reaction.

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