Stimulation of phospholipase A_2 activity by high osmotic pressure on cholesterol-containing phospholipid vesicles

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The hydrolytic action of porcine pancreatic phospholipase A_2 (PLA_2) on cholesterol-containing dipalmitoylphosphatidylcholine liposomes was studied under various osmotic conditions by means of the phase transition release (PTR) technique. A reduced rate in the release of carboxyfluorescence (CF) from the cholesterol-containing liposomes was observed with treatment by PLA_2 concomitant with the decrease in its enzymatic activity. However, the reduced PLA_2 activity was elevated about 5-fold in medium of high osmotic pressure. This evidence suggests that high osmotic pressure might be responsible for the molecular packing at the bilayer surface or the high curvature of liposomal membrane structure.

Phospholipase A_2 *Liposome* *Cholesterol* *Osmolarity* *Phase transition* *Bilayer curvature*

1. INTRODUCTION

Activation of membranous phospholipase A_2 (PLA_2) is believed to be an important step in metabolic stimulation of non-excitable cells [1]. Although it seems apparent that phospholipase activities depend on the physicochemical state of substrate phospholipids, the mechanism by which their catalytic activities are modulated on a lipid–water interphase is still obscure. Among various lipases, pancreatic PLA_2 has a unique temperature dependency, the enzyme shows maximum activity at the temperature range in which the substrate lipids undergo thermotropic transition [2]. Irregularities in the packing of lipid molecules at the border of the liquid-crystalline phase on the plane of the lipid bilayer have been thought to favor the enzyme–substrate interactions [3]. Thus, PLA_2 may represent boundaries between different lipid domains within bilayers (structurally defective bilayers) which are formed at the phase transition temperature. Defects of such structural organization are present at the liquid-crystalline phase transition and in lipid bilayers with strong curvature [4].

Cholesterol is an important constituent in various plasma membranes and modulates physicochemical states of membranous phospholipids [5]. When phospholipids are in the liquid-crystalline state, cholesterol reduces the motional freedom of fatty acyl chains [6], which results in a decreased mean molecular area of phospholipids [7]. In contrast, cholesterol increases chain motility when lipids are in the gel state [6]. Thus, cholesterol inhibited membranous PLA_2 activity presumably due to elimination of the phase transition of phospholipid vesicles [3].

Recently, we reported a new method for the measurement of PLA_2 activity by means of the phase transition release technique [8]. Using this method, liposomal PLA_2 was shown to be activated by high concentrations of ions such as NaCl. In an attempt to understand the mechanism of this activation, the effects of high osmolarity on liposome structure and enzyme activity were studied. This work shows that stimulation of PLA_2

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activity on liposomes is closely correlated with the formation of strong curvature in the liposomal bilayer.

2. MATERIALS AND METHODS

2.1. *Reagents*

DL-α-Dipalmitoylphosphatidylcholine (DPPC) and cholesterol were purchased from Sigma. Porcine pancreatic PLA_2 (800 IU/ml) was supplied by Boehringer. Carboxyfluorescein (CF) was obtained from Kodack and further purified as in [9]. All reagents used were of analytical grade.

2.2. *Liposomes*

Liposomes were made of DPPC or DPPC–cholesterol (1:0–1:0.3 molar ratio) and unilamellar liposomes of DPPC containing CF were prepared as in [8]. CF-containing DPPC–cholesterol liposomes were fractionated by Sepharose 4B and fractions eluted in the same position as those for unilamellar DPPC liposomes [8] were used for the present experiments. All liposomes were prepared in 20 mM sodium phosphate buffer (pH 6.8), which contained 0.1 M NaCl and CF.

2.3. *Phase transition release*

Phase transition release (PTR) was measured as in [10]. Liposomes were suspended in 20 mM phosphate buffer (pH 6.8) in the presence of 0.2–1.0 M NaCl or 0.2–1.0 M sucrose.

2.4. *Determination of lysophosphatidylcholine*

Liposomes were incubated at 37°C for 10 min in the presence or absence of PLA_2 (10 μg/ml). PLA_2-generated lysophosphatidylcholine (lyso-PC) was separated as in [11]. Protein was measured as described in [12] using bovine serum albumin as standard.

2.5. *Osmolarity*

Osmolarity of the incubation medium was determined by an Advanced Instruments osmometer.

2.6. *Differential scanning calorimetry*

Calorimetric experiments were performed on a Rigaku DSC-8230 apparatus operating at a heating rate of 5°C/min. Twenty-five μl of DPPC or DPPC-cholesterol liposomes were suspended in 20 mM phosphate buffer (pH 6.8), containing 0.1–1.0 M NaCl, and sealed in aluminum pans. Phosphate buffer (20 mM, pH 6.8) which contained 0.1 M NaCl was used as a reference solution. Differential scanning calorimetry thermograms were obtained in the temperature range 0–70°C.

2.7. *Electron microscopic observations*

Morphological studies of liposomes in various osmotic media were performed by the negative staining method in [13], and specimens were observed in a Hitachi electron microscope model HS300.

3. RESULTS AND DISCUSSION

3.1. *Effect of medium osmolarity*

It has been known that hydrolysis of phosphatidylcholine by PLA_2 occurs only in the temperature range of the phase transition [3]. Thus, in the

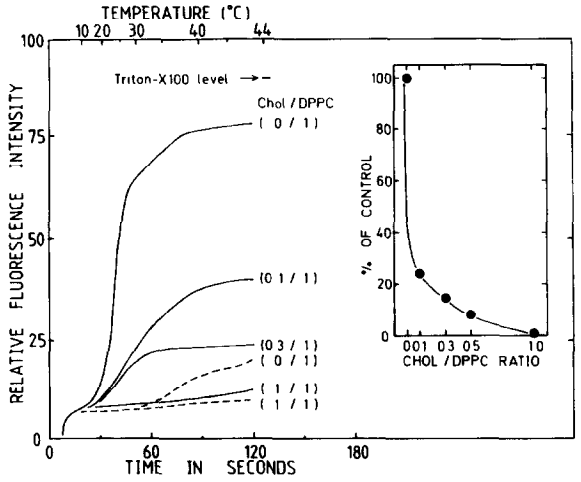

Fig.1. Phase transition release of CF from DPPC–cholesterol liposomes by PLA_2. CF-containing liposomes containing different ratios of cholesterol/DPPC were incubated with PLA_2 (1 μg/ml) in a medium of 0.1 M NaCl–20 mM sodium phosphate buffer (pH 6.8) and 1 mM $CaCl_2$. Fluorescence intensity of CF at 515 nm was recorded by 470 nm excitation using a Shimadzu spectrofluorometer. Total fluorescence intensity of CF in the incubated liposomes was measured after addition of 0.1% Triton-X 100. (——) With PLA_2 (1 μg/ml), (– – –) without PLA_2. Inset: percent inhibition of CF release by the increased concentration of cholesterol in liposomes

presence of 10–50% cholesterol in phospholipid vesicles, CF release from liposomes occurred at slower rates than in the absence of cholesterol (fig.1). In the presence of over 30% of vesicular cholesterol content, CF release was markedly inhibited at any temperatures tested (0–50°C). Incubation of cholesterol-containing (30%) phosphatidylcholine vesicles resulted in a PLA_2-induced CF release; the rate of release around the phase transition temperature increased with high concentration of sucrose (fig.2A). The rate of CF release was maximum at 0.8 M sucrose concentration which is equivalent to 1334 mosmol/kg. Osmolarity-dependent CF release occurred similarly in media with increasing concentrations of NaCl (fig.2B). Thus, CF release by high sucrose concentration might reflect activation of liposomal PLA_2 by high osmotic pressure. Fig.3 shows the osmotic dependency of PLA_2 activity on DPPC–cholesterol vesicles. Lyso-PC formation by PLA_2 was increased by high concentration of either sucrose or NaCl. Lyso-PC formation at different sucrose concentrations occurred quite similarly to that observed with NaCl. These results might suggest that PLA_2 activity associated with cholesterol–phospholipid vesicles was enhanced by high medium osmolarity presumably due to the increase in bilayer curvature of substrate liposomes.

3.2. *CF release from liposomes by lyso-PC*

It seemed that CF release from DPPC–cholesterol vesicles at high osmotic pressure might be caused by lyso-PC which was formed by PLA_2. To test this possibility, the effect of lyso-PC on CF release was measured at different medium osmolarities. As shown in fig.4A, CF release from DPPC–cholesterol liposomes was induced by lyso-PC. This phase transition release also required high medium osmolarity, CF release was maximum at 1500 mosmol/kg. No detectable CF

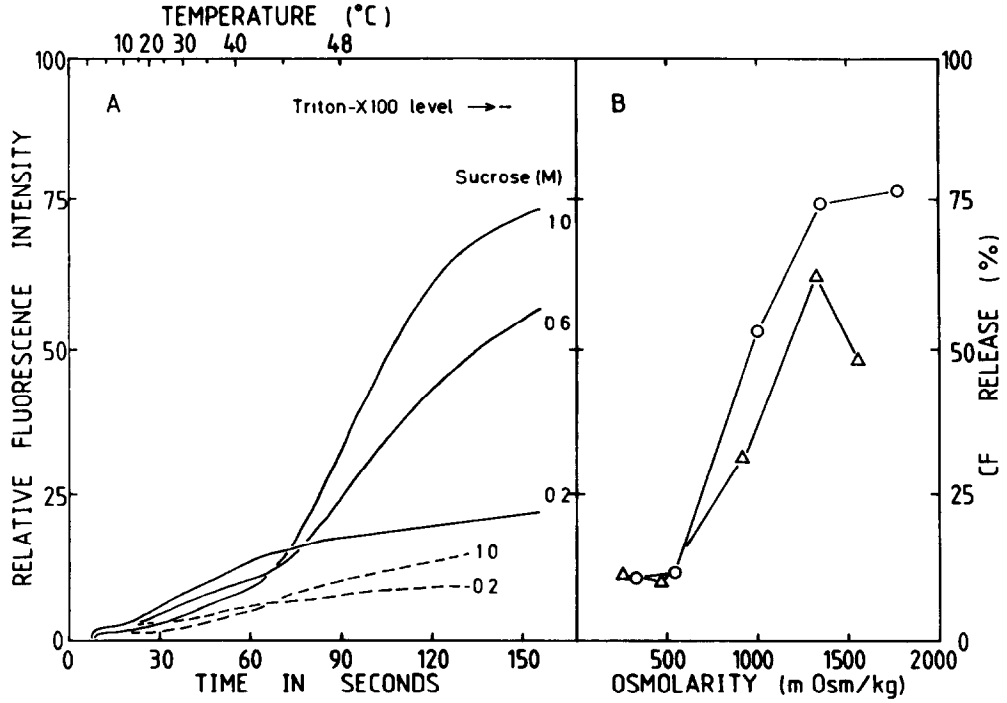

Fig.2. Effect of different concentrations of NaCl or sucrose on the phase transition release of CF from DPPC–cholesterol liposomes by PLA_2. (A) DPPC–cholesterol (1:0.3) liposomes were incubated in media of various concentrations of sucrose containing 20 mM sodium phosphate buffer (pH 6.8), 1 mM $CaCl_2$ and PLA_2 (10 μg/ml) at 0°C and transferred to the cuvette holder at 50°C. (——) With PLA_2 (10 μg/ml), (– – –) without PLA_2. (B) Changes in percent release of CF by the increase in osmolarity with sucrose or NaCl. Experimental conditions were as in A. (○) With different concentrations of sucrose, (△) with different concentrations of NaCl.

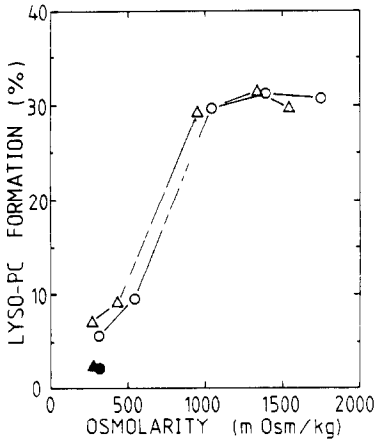

Fig.3. Hydrolysis of DPPC–cholesterol liposomes by PLA_2 at different osmolarities of the incubation mixture. DPPC liposomes (750 μM) containing 30% cholesterol were incubated at 42°C for 10 min in a medium of 20 mM sodium phosphate buffer (pH 6.8), 1 mM $CaCl_2$, PLA_2 (10 μg/ml) and different concentrations of sucrose or NaCl. (○) With different concentrations of sucrose, (△) with different concentrations of NaCl. (○, △) Formed lyso-PC without PLA_2 in isotonic sucrose or NaCl solution.

release was induced by lyso-PC in an isotonic medium. Fig.4B depicts the CF release as a function of different medium osmolarity. Stimulation of CF release occurred similarly to that of PLA activity (see fig.2). However, the temperature dependency of lyso-PC-induced CF release was different from that induced by enzyme. Lyso-PC-induced CF release occurred maximally at 38°C, while CF release by PLA_2 (10 μg/ml) increased progressively with increasing temperatures from 30 to 50°C (not shown). These results suggest that the phase transition release of CF depends preferentially on lyso-PC which was generated by PLA_2. Of course, the enzyme activity was the rate-limiting step for CF release. Thus, the formation of lyso-PC by PLA_2

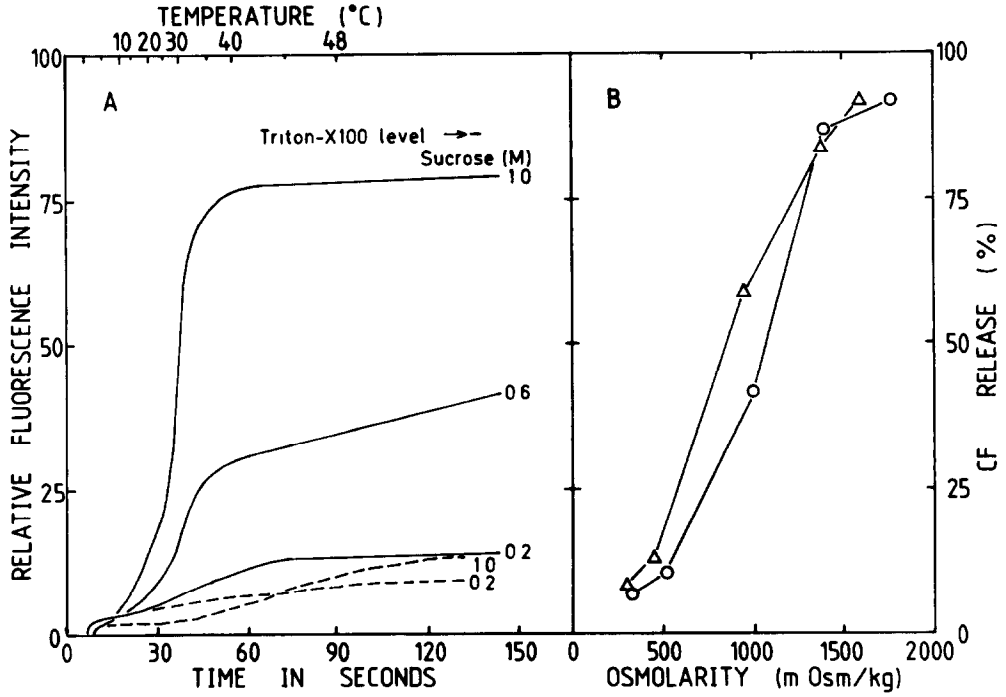

Fig.4. Phase transition release of CF from DPPC–cholesterol liposomes by lyso-PC at different osmolarities of incubation mixture. (A) Experimental conditions were as in fig.3 except the lyso-PC used was 25 μM instead of PLA_2. (——) With lyso-PC, (– – –) without lyso-PC. (B) Changes in percent release of CF by lyso-PC at different osmolarities of the incubation mixture at 48°C. Experimental conditions were as in A. (○) With different concentrations of sucrose, (△) with different concentrations of NaCl.

resulted in CF release from DPPC–cholesterol liposomes under high medium osmolarity.

3.3. *Thermotropic transition of liposomes*

PLA_2 activity has been known to pass through a maximum in temperature range in which substrate lipids undergo their thermotropic transition [3]. At higher cholesterol concentrations, a liquid-condensed packing of bilayer lipids is reached and the cooperative phase transition from the liquid-crystalline to gel state is abolished [6]. Thus, introduction of cholesterol molecules into the phospholipid bilayer may inhibit PLA_2 activity with concomitant reduction of the energy content of the phase transition [5]. However, the enzyme activity in DPPC–cholesterol vesicles (1:0.3 in molar ratio) was increased by high medium osmolarity. As shown in fig.5, the thermotropic transition of DPPC vesicles is markedly reduced by increasing cholesterol content. This behavior of DPPC vesicles is not changed by increasing the medium osmolarity, suggesting that no phase transition occurred at high osmotic pressure.

3.4. *Effect of medium osmolarity on liposomal structure*

Kinetic analysis revealed that the enzyme activity can be modulated by perturbation of the molecular arrangement of the enzyme and substrate lipids in the liposomal bilayer. To gain molecular insight into the mechanism by which PLA_2 activity is controlled, electron microscopic observation of liposomes was performed under different osmotic conditions. As shown in fig.6, the strongly curved

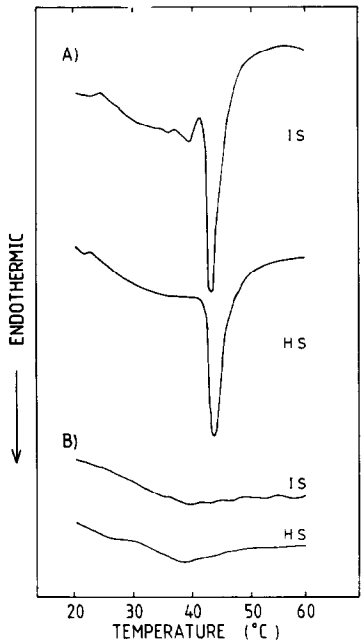

Fig.5. Differential calorimetric scans of DPPC and DPPC–cholesterol liposomes in medium of isotonic and hypertonic solutions. Liposome concentration used was 15 mM DPPC in 0.1 M NaCl, 20 mM sodium phosphate buffer (pH 6.8) and the cholesterol/DPPC ratio of the liposomes was 0.3. Differential scanning calorimetry spectra were obtained between 0 and 70°C. (A) DPPC liposomes. (B) DPPC–cholesterol liposomes. I.S., isotonic: 0.1 M NaCl–20 mM sodium phosphate buffer (pH 6.8). H.S., hypertonic: 0.8 M NaCl–20 mM sodium phosphate buffer (pH 6.8)

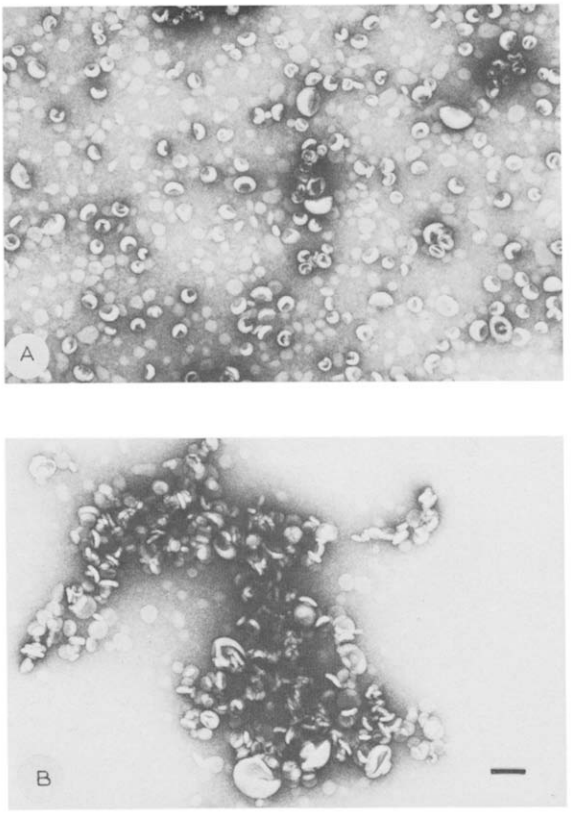

Fig.6. Electron micrographs of DPPC–cholesterol liposomes in isotonic and hypertonic solutions. (A) Isotonic condition [0.2 M sucrose–20 mM sodium phosphate buffer (pH 6.8)]. (B) Hypertonic condition [0.8 M sucrose–20 mM sodium phosphate buffer (pH 6.8)]. Bar, 100 nm; ×27200.

bilayer of DPPC–cholesterol liposomes was seen at high sucrose concentrations. This might suggest that increased PLA_2 activity under high osmotic pressure is due to formation of a strongly curved bilayer by which substrate lipids become more accessible to the enzyme.

Together with PLA_2 activation in the narrow temperature range centered at the melting temperature of substrate lipids [14], the present results indicate the significant participation of lipid bilayer curvature in apparent enzyme activity towards liposomal substrates at their phase transition temperature. The proposal [15] that the packing defect in a highly curved lipid bilayer in the gel state [4] might be the main factor in the relatively prompt formation of the enzyme–substrate complex is consistent with the present findings.

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