Permeability changes of phospholipid liposomes caused by pancreatic phospholipase A_2: analysis by means of phase transition release

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1. INTRODUCTION

Many biological messages are recognized by the binding of the ligands to specific receptors of the outer surface of the cell membrane [1–4]. These bindings then initiate certain chemical and physical changes in the membrane. One of the early changes in membrane state is the activation of phospholipase A_2 (PLA_2) [5]. This reaction has many important functions such as in biosynthesis of prostaglandins, in physicochemical changes of biomembranes and in regulation of the activities of other membrane bound enzymes. Therefore, the activation of membrane bound PLA_2 has a key role in the mechanisms of cell activation. One of the important features of the regulation of PLA_2 is the dependency of activation on membrane structure. Many investigations were carried out on the analogies with much better defined systems using soluble phospholipase, though this system is essentially different from those occurring in natural membranes. In those studies, it was proposed that a particular region of PLA_2, so called interface recognition site, is involved in interaction with phospholipid structure [6]. Moreover, it was considered that phosphatidylcholine can be hydrolysed only near the transition temperature where lipid in liquid crystalline phase and in the gel phase coexist [7,8].

We have therefore applied the phase transition release technique to the study of the activation mechanism as related to the changes in physiological characteristics of the membrane and bring evidence that the maximal carboxyfluorescein release which was observed below 38°C by PLA_2 corresponds to phase transition point of dipalmitoyl phosphatidylcholine.

2. MATERIALS AND METHODS

2.1. *Reagents*

DL-α-Dimyristoyl phosphatidylcholine (DMPC) and DL-α-dipalmytoyl phosphatidylcholine (DPPC) were purchased from Sigma Chemical Co. Phospholipase A_2 from porcine pancreas (800 IU/mg) was supplied by Boehringer. All chemicals used were of analytical grade and water was deionized and distilled.

2.2. *Vesicles*

Liposomes were made with DPPC by the method described previously [9]. Unilamellar vesicles containing carboxyfluorescein (CF) were prepared by the method of Klausner et al. [10]. Lipids (15 μmol/ml) were dissolved in chloroform–methanol (2:1). One ml of DPPC solution was evaporated and lyophylized in a test tube. The lipid (15 μmol) was hydrated by vortex-mixing for 15 min in 1 ml of 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) containing 0.1 M carboxyfluorescein (CF) at 50–55°C. The vesicles were sonicated at 45–52°C for 60 min in a 15 ml test tube using a Branson sonifier (Type 185). The sample became optically clear within 2–5 min and 1.5 ml were applied to a Sepharose 4B column (1.5 × 25 cm). The column was eluted at 4°C with buffer containing 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) and

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small unilamellar vesicles were fractionated to supply the phase transition release experiment.

2.3. *Phase transition release (PTR)*

The PTR curve was obtained by the method of Weinstein et al. [11]. The unilamellar liposome suspension was placed in an ice cold small cuvette and transferred to the heated (50°C) small chamber of a Shimadzu fluorospectrophotometer (Type RF-510). The temperature of the cuvette holder was maintained by a water bath and monitored by a digital temperature thermister kept above the light path in the solution of cuvette. In this case the liposome suspension in the original cuvette was mixed with a magnetic stirrer. The CF was excited at 470 nm and emission was read at 515 nm. To determine the fluorescence intensity achieved by the release of 100% of the dye from the liposomes, 5 μl of 10% Triton X-100 solution was added to dissolve the vesicles. For the effect of PLA_2 on the PTR, PLA_2 was added to the liposome suspension in the medium of 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) at 4°C in the presence or absence of EGTA (1 mM) or Ca^{2+} (1 mM).

2.4. *Association of PLA_2 with the liposomes*

In the presence of 1 mM EGTA, PLA_2 was incubated for 5 min with DPPC liposomes at 4, 22, 38 and 43°C. The liposomes were separated from PLA_2 by Ficoll density gradient centrifugation at each temperature as described in the previous paper [9]. The PLA_2 associated liposomes were detected by SDS polyacrylamide gel electrophoresis. The relative amount of associated PLA_2 was estimated using a Shimadzu dual beam gel scanner (Type CS-900).

3. RESULTS AND DISCUSSION

3.1. *Increase in PTR of CF by porcine pancreatic PLA_2*

A change of fluorescence intensity through the phase transition point of DPPC liposome was confirmed as shown in fig.1. The PTR curve was changed by addition of a small amount of PLA_2 and the change depended on the concentration of added enzyme in the presence of 1 mM $CaCl_2$ as indicated in fig.1. The requirement for maximum fluorescence change was about 0.05 μg/ml. But in the presence of 1 mM EGTA, a potent inhibitor of

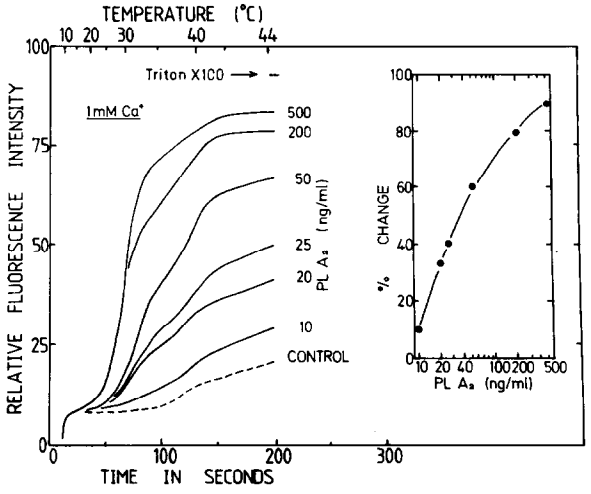

Fig.1. The effect of the enzymatic degradation of phospholipid by PLA_2 on the PTR of CF from DPPC liposomes and its concentration dependency. CF-containing unilamellar DPPC liposomes were incubated with various concentrations of pancreatic PLA_2 in the medium of 0.1 M NaCl–20 mM phosphate buffer (pH 6.8), 1 mM $CaCl_2$ at 0°C and transferred to the cuvette holder at 50°C. Fluorescence intensity at 515 nm was recorded by excitation with 470 nm using a Shimadzu spectrofluorometer. Total fluorescence intensity of CF in the incubated liposomes was measured after addition of Triton X-100. For the half maximal CF release by enzyme activity, 50 ng/ml of PLA_2 was required. Inserted figure shows the per cent change of CF release by the treatment with PLA_2 in the presence of $CaCl_2$.

PLA_2 due to its chelating action of Ca^{2+}, the required amount of enzyme for maximum change was increased remarkably to 0.6 mg/ml (fig.2). Namely this result shows that the change in the PTR curve in the presence of PLA_2 and Ca^{2+} is due to the enzymatic activity of PLA_2 and that the changes in the PTR curve by PLA_2 in the presence of EGTA may be the result of the integration of the enzyme protein into the lipid layer of liposomes as was observed in the case of actin or tubulin [10,12].

The complicated pattern of CF release from liposomes incubated with PLA_2 at different constant temperatures is shown in fig.3. But the highest velocity of CF release was observed below the phase transition point, 15–25°C as indicated in fig.3.

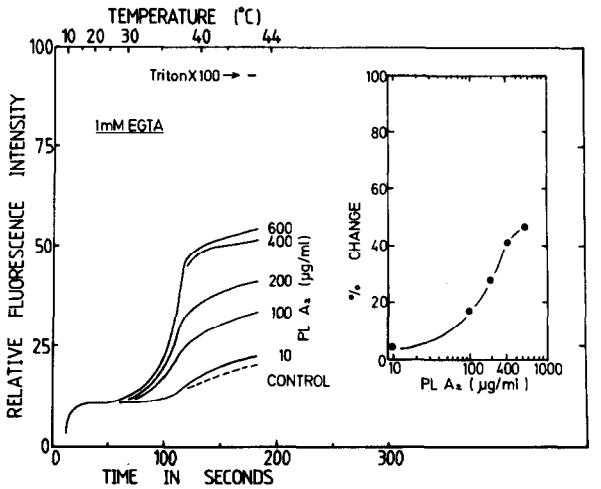

Fig.2. The effect of PLA_2 protein on the phase transition release curve of CPPC liposomes. Experimental conditions were as in fig.1 except the increased enzyme concentration and the substitution of EGTA (1 mM) for $CaCl_2$ in the incubation medium. The requirement for enzyme protein for half-maximal CF release was about 1 mg/ml. Inserted figure shows the per cent change of CF release by the treatment with PLA_2 in the absence of $CaCl_2$.

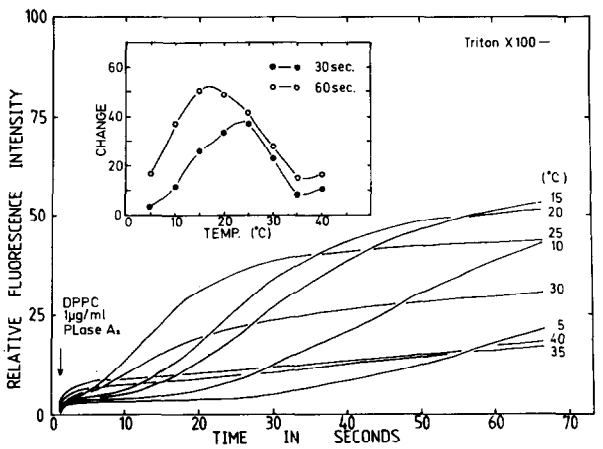

Fig.3. The effect of PLA_2 on the release of CF from DPPC liposomes at various temperatures. Unilamellar DPPC liposomes containing CF were incubated with PLA_2 (1 μg/ml) in a medium of 0.1 M NaCl–20 mM phosphate buffer (pH 6.8) at various temperatures. The degree of CF release 30–60 s after incubation was plotted against temperature. High activity of PLA_2 was detected over the range of 15–30 °C.

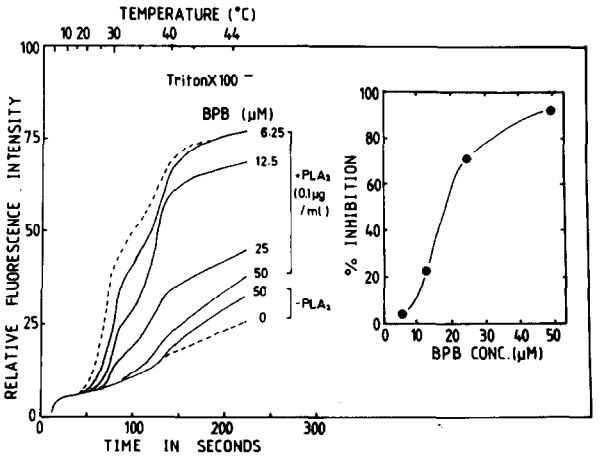

Fig.4. The effect of PLA_2 inhibitor on the phase transition release curve of DPPC in the presence of PLA_2 and $CaCl_2$. Experimental conditions were as in fig.1. The inhibitor of PLA_2 was added to the medium before incubation. Bromophenacylbromide (BPB), a potent inhibitor of PLA_2, inhibited the release of CF from DPPC liposomes depending on its concentration. The degree of inhibition was expressed in per cent change of CF release by the addition of BPB. The concentration for half-maximal inhibition was about 30 μM. Inserted figure shows the per cent inhibition of PLA_2 induced CF release by the addition of BPB.

3.2. *Effect of PLA_2 inhibitors on the PTR curve of DPPC liposomes*

Recently two kinds of PLA_2 inhibitors were reported [13]. One class of inhibitors is drugs which react with PLA_2 directly such as mepacrine, phentermine and bromophenacyl bromide (BPB) and the other class are drugs which modify membrane fluidity or lower the transition temperature such as anesterics, ethrane and trichloroethylene. In the experimental system described above, both classes of PLA_2 inhibitors inhibit release of CF from liposomes by PLA_2 activity below the temperature of phase transition (fig.4). The phase transition point of DPPC liposomes was shifted to a lower temperature by the addition of 0.1% (50 μM) trichloroethylene and the CF release induced by PLA_2 was completely inhibited by the drug at 0.1% (data was not shown). In contrast to this, BPB had no effect on the phase transition point as shown in

Table 1

Association of PLA_2 with DPPC liposomes at various temperatures

Temp. (°C)	Liposome layers (%)				Sample zone
	L_0* (1.00–1.01)	L_1 (1.01–1.03)	L_2 (1.03–1.04)	L_3 (1.04–1.05)	P (1.06)
4	–**	0***	0	57.4	42.6
22	–	12.5	20.8	22.2	44.4
38	–	0	0	17.7	82.3
43	0	–	–	–	100.0

* Interface liposome layer (L_0, L_1, L_2 or L_3) is 1.00–1.01, 1.01–1.03, 1.03–1.04 or 1.04–1.05 specific gravity (g/ml) of Ficoll 400 respectively.

** No existence

*** Non-associated liposomes

Pancreatic PLA_2 (500 μg/ml) was incubated with DPPC liposomes (15 μmol/ml) at 4, 23, 38 and 43°C for 5 min and protein associated liposomes were separated from incubation mixture by Ficoll density gradient centrifugation (100 000 × *g* for 60 min) at each temperature. The numbers express the per cent of protein distribution.

fig.4. The requirement of BPB for the inhibition of PTR induced by PLA_2 was the same order of concentration as that described by several investigators using other biochemical methods.

3.3. *Association of PLA_2 with DPPC liposomes*

As described in the previous section, PLA_2 activity was observed under conditions where gel and liquid crystal states of liposomes lipid coexist. To clarify whether PLA_2 associates with liposomes under same conditions where PLA_2 activity is exhibited, the Ficoll density gradient centrifugation technique was applied to separate the enzyme-associated liposomes from the incubation mixture after the incubation of PLA_2 with DPPC liposomes in the presence of EGTA (1 mM). As indicated in table 1, PLA_2 associated with liposomes and separated from the reaction mixture at temperatures where enzyme activity can be detected. Even at 4°C PLA_2 was associated but no association was observed over 38°C. Namely, it is suggested that the PLA_2 can associate with liposomes even at 4°C without integration but the integration into the liposomal lipid layer proceeded in the presence of gel and liquid crystal phases as in the case of actin or tubulin [12].

From these experimental data we can conclude that the PLA_2 can interact with substrate in liposomes under a lower temperature than the phase transition point and can integrate into the phospholipid membrane layer under the condition where gel and liquid crystalline phases coexist. Furthermore, in this experiment it was determined that the PTR method is a useful technique for studying the membrane–PLA_2 interaction and that the activity of very low concentration of PLA_2 can be measured by using the PTR technique.

REFERENCES

[1] Flaherty, J.T.O. and Ward, P.A. (1978) Seminars in Hematol. 16, 163–174.

[2] Utsumi, K., Sugihama, K., Miyahara, M., Naito, M., Arai, M. and Inoue, M. (1977) Cell Struct. Funct. 2, 203–209.

[3] Utsumi, K., Miyahara, M., Okimasu, E., Sugiyama, K. and Inoue, M. (1979) Phys. Chem. Phys. 11, 365–369.

[4] Watanabe, S., Morimoto, Y.M., Shiraishi, N., Sano, A. and Utsumi, K. (1981) Cell Struct. Funct. 6, 263–267.

[5] Hirata, F., Corcoran, B.A., Venkatasubramanias, K., Schiffman, E. and Axelrod, J. (1979) Proc. Natl. Acad. Sci. USA 76, 2640–2643.

[6] Pieterson, W.A., Vidal, J.C., Volwerk, J.J. and Hass, B.H. (1974) Biochemistry 13, 1455–1469.

[7] Op Den Kamp, J.A.F., Degier, J. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 345, 253–256.
[8] Op Den Kamp, J.A.F., Kauerz, M. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 169–177.
[9] Utsumi, K., Okimasu, E., Takehara, Y., Watanabe, S., Miyahara, M. and Moromizato, Y. (1981) FEBS Lett. 124, 257–260.
[10] Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R. and Flavin, M. (1981) J. Biol. Chem. 256, 5879–5885.
[11] Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E. and Blumenthal, R. (1981) Biochim. Biophys. Acta 647, 270–284.
[12] Utsumi, K., Okimasu, E., Morimoto, Y.M., Nishihara, Y. and Miyahara, M. (1982) FEBS Lett., in press.
[13] Vigo, C., Lewis, G.P. and Piper, P.J. (1980) Biochem. Pharmac. 29, 623–627.