Modulation of phospholipase A₂ activity by the tumour promoters phorbol esters and teleocidin

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The effects of tumour promoters, namely phorbol esters and teleocidin, on the activity of porcine pancreatic phospholipase A₂ (PLA₂) was investigated by using a system of small unilamellar vesicles composed of dipalmitoylphosphatidylcholine (DPPC). DPPC vesicles encapsulating Quin 2 (Quin 2/DPPC vesicles) were suspended in a medium containing Ca²⁺. The addition of PLA₂ to Quin 2/DPPC vesicles increased the fluorescence intensity of Quin 2. This increase was due to chelation of Quin 2 with Ca²⁺, which resulted from an increase in the permeability of the phospholipid bilayer caused by the hydrolytic activity of PLA₂. The tumour promoters phorbol 12-myristate 13-acetate (PMA) and teleocidin, at low concentrations, enhanced PLA₂ activity at temperatures below the phase-transition temperature of the membrane, but, in contrast, high concentrations of the tumour promoters suppressed PLA₂ activity. Phorbol 12-myristate (PM) also had a similar effect on PLA₂ activity. PMA and PM disturbed the membrane structure markedly, which was indicated by the enhanced leakage of carboxyfluorescein (CF) from DPPC vesicles encapsulating CF. On the other hand, phorbol 12,13-didecanoate and 4 α -phorbol 12,13-didecanoate, which did not disturb the membrane structure to the same extent, had an insignificant effect on PLA₂ activity. It is therefore concluded that PLA₂ catalyses the hydrolysis of phospholipids in bilayer vesicles which contain a moderate degree of structural defects. However, the effects of tumour promoters on PLA₂ activity was not related to their potencies as inflammatory and tumour-promoting agents.

INTRODUCTION

Phorbol 12-myristate 13-acetate (PMA), teleocidin and aplysinatoxin are potent tumour promoters, as shown in a two-step carcinogenesis experiment on mouse skin (Hecker, 1978; Fujiki *et al.*, 1984a,b). These tumour promoters are known to possess pleiotropic effects, including the inflammatory reaction which is shown by an irritation test on mouse ear. These tumour promoters have also been shown to activate protein kinase C (Castagna *et al.*, 1982; Parker *et al.*, 1984), which plays a key role in cellular signal transduction.

Cellular phospholipase A₂ (PLA₂) may be involved in the inflammatory process, as activation of PLA₂ increases the release of arachidonic acid, a precursor of prostaglandins (Ross *et al.*, 1985; Dennis, 1987; Nakashima *et al.*, 1987). Tumour promoters stimulate prostaglandin E₂ production (Ohuchi *et al.*, 1985, 1986, 1987a) and arachidonic acid metabolism (Levine & Fujiki, 1985; Levine *et al.*, 1986; Ohuchi *et al.*, 1987b). Although PMA and teleocidin have been postulated to be incorporated into the binding site of PLA₂ (Smythies, 1980), the effects of tumour promoters on PLA₂ activity have not been studied.

Although the mechanism of action of PLA₂ has not yet been clarified, the hydrolysis of lecithin by PLA₂ is enhanced by the presence of small structural irregularities, called 'cracks', in the lipid bilayer membrane (Upreti & Jain, 1980; Slotboom *et al.*, 1982). Tumour promoters such as PMA and teleocidin are thought to bind to the bilayer membrane and to cause changes in membrane structure (Nam *et al.*, 1989a). For example, PMA induces the leakage of carboxyfluorescein (CF) through the lipid bilayer membrane, and teleocidin decreases membrane fluidity at temperatures below the phase-transition temperature.

This paper reports the effects of tumour promoters on porcine pancreatic PLA₂ activity by using a system of small unilamellar vesicles composed of dipalmitoylphosphatidylcholine (DPPC).

The PLA₂ activity was determined by fluorescence enhancement, which was induced by the change in membrane permeability caused by catalytic decomposition of lipid molecules by PLA₂.

EXPERIMENTAL

Materials

DPPC, CF and porcine pancreatic PLA₂ were purchased from Sigma. PMA phorbol 12-myristate (PM), phorbol 12,13-didecanoate (PDD), and 4 α -phorbol 12,13-didecanoate (4 α PDD) were obtained from LC Services Inc., MA, U.S.A. Quin 2 was purchased from Dojin Inc. (Kumamoto, Japan). Teleocidin was isolated as previously described (Fujiki & Sugimura, 1982). The highest available grades of reagents were used.

Preparation of DPPC vesicles encapsulating Quin 2

DPPC vesicles encapsulating Quin 2 were prepared as described previously (Kimura *et al.*, 1989). DPPC was dispersed in Hepes, pH 7.4, containing 0.1 M-NaCl and 20 mM-Quin 2, sonicated and centrifuged at 100 000 g. The DPPC vesicles were then eluted through a G-50 column to remove free Quin 2. The vesicles obtained were suspended in Hepes buffer (0.14 mM-DPPC; Quin 2/DPPC vesicles), and fluorescence spectra were measured following indicated additions with moderate stirring. The stock solutions of tumour promoters were prepared in ethanol solution, and were added so that the organic solvent concentration was less than 1% of the total volume. Normally, Quin 2/DPPC vesicles [0.14 mM-DPPC (final concentration), 45 μ l] were incubated with tumour promoter in buffer (2.1 ml) containing Ca²⁺ (10 mM). PLA₂ was added to the suspension and the fluorescence intensity of Quin 2 was monitored. In an experiment examining the possibility of a direct interaction of a tumour promoter with PLA₂, the tumour promoter and PLA₂ were preincubated in

Abbreviations used: CF, 5/6-carboxyfluorescein; DPPC, dipalmitoylphosphatidylcholine; PDD, phorbol 12,13-didecanoate; 4 α PDD, 4 α -phorbol 12,13-didecanoate; PM, phorbol 12-myristate; PLA₂, porcine pancreas phospholipase A₂; PMA, phorbol 12-myristate 13-acetate.

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buffer (2.1 ml) containing Ca^{2+} (10 mM), and then Quin 2/DPPC vesicles were added.

Temperature was controlled by using a thermostated water jacket. Excitation and monitoring wavelengths of Quin 2 were 339 and 510 nm respectively. Fluorescence measurements were carried out on an MPF-4 spectrophotometer (Hitachi, Tokyo, Japan).

Distribution of PLA_2 in DPPC vesicles

Distribution of PLA_2 in DPPC vesicles was examined by the emission spectrum of PLA_2 according to the method reported by Jain *et al.* (1982). Fluorescence spectra of PLA_2 were measured in a buffer solution (Hepes, 10 mM, NaCl, 0.1 M) containing DPPC vesicles. An excitation wavelength of 275 nm was used.

CF leakage

DPPC vesicles encapsulating CF (CF/DPPC vesicles) were prepared according to the method reported by Barbet *et al.* (1984). The CF/DPPC vesicles were suspended in 5 mM-Tris/HCl (pH 7.4) containing 0.1 M-NaCl, and the tumour promoter was added. Excitation and monitoring wavelengths of CF were 470 and 520 nm respectively.

RESULTS AND DISCUSSION

Hydrolysis of Quin 2/DPPC vesicles by PLA_2

The hydrolytic activity of PLA_2 has been studied previously by various methods, which can be classified into two groups: fixed-time methods and time-resolved methods. The fixed-time method, using radiolabelled phospholipids, is convenient for detection of PLA_2 , but is not suitable for analysis of the mechanism of PLA_2 activity, because the hydrolytic reaction catalysed by PLA_2 cannot be characterized by a simple rate constant due to the presence of a latency period in the time course of the reaction. On the other hand, the time-resolved methods such as titrimetry (Tinker *et al.*, 1978) and spectrophotometry using fluorescent phospholipids (Thuren *et al.*, 1984) provide information on the time course of PLA_2 activity. However, titrimetry cannot avoid

difficulties in measuring pH changes on addition of reagents, due to the inaccessibility of buffer reagents. The use of fluorescent phospholipids is also disadvantageous because they disturb the membrane structure on incorporation into the natural lipid membrane, and the activity of PLA_2 is very sensitive to fluctuations in the membrane structure.

In the present investigation, in order to eliminate the latter difficulty of the time-resolved method, the fluorescent probe Quin 2 was encapsulated in DPPC vesicles and the Quin 2/DPPC vesicles were suspended in medium containing Ca^{2+} or Ba^{2+} . PLA_2 activity was measured continuously by the increase in fluorescence intensity of Quin 2 upon chelation with Ca^{2+} or Ba^{2+} (Tsien, 1980), which resulted from the change in membrane permeability caused by the hydrolysis of lipid molecules by PLA_2 .

Addition of PLA_2 to Quin 2/DPPC vesicles increased the fluorescence intensity of Quin 2 at temperatures below the phase-transition temperature of the membrane (Fig. 1). This increase in the fluorescence intensity of Quin 2 is confirmed to be the result of the hydrolytic activity of PLA_2 by the following observations. (1) The time course of the reaction of PLA_2 with DPPC vesicles was similar to that determined by another method previously reported (Menashe *et al.*, 1986). (2) At temperatures above the phase-transition temperature of the membrane, and in the presence of Ba^{2+} instead of Ca^{2+} , where hydrolysis cannot take place (Roholt & Schlamowitz, 1961; Menashe *et al.*, 1986), addition of PLA_2 did not increase the fluorescence intensity of Quin 2. (3) PLA_2 modified at His-48 (Volwerk *et al.*, 1974; Roberts *et al.*, 1977) did not alter significantly the fluorescence intensity of Quin 2.

Effects of tumour promoters on PLA_2 activity

The effect of PMA on the activity of PLA_2 was studied at various concentrations (Fig. 1). PMA was incubated with Quin 2/DPPC vesicles prior to the addition of PLA_2 . At concentrations lower than $3.1 \mu\text{M}$ -PMA, the fluorescence intensity of Quin 2 showed a steep increase, whereas it showed only a moderate increase at PMA concentrations higher than $5.2 \mu\text{M}$. A concentration of $10.4 \mu\text{M}$ -PMA gave a curve similar to that without PMA. Teleocidin showed effects similar to those of PMA on

Fig. 1. Time course of the change in fluorescence intensity of Quin 2 as a function of PMA concentration on addition of PLA_2 at 30 °C

Numbers represent the following concentrations of PMA added to the Quin 2/DPPC vesicles: 0, 0 μM ; 1, 1.0 μM ; 2, 2.1 μM ; 3, 3.1 μM ; 4, 5.2 μM ; 5, 7.8 μM ; 6, 10.4 μM . PMA was preincubated with Quin 2/DPPC vesicles and then PLA_2 was added. [DPPC] = 0.14 mM, [PLA_2] = 71 nM and [Ca^{2+}] = 10 mM. The broken line shows the effects of the addition of PMA (3.1 μM) in the absence of PLA_2 .

Fig. 2. Time course of the change in fluorescence intensity of Quin 2 as a function of teleocidin concentration on addition of PLA_2 at 30 °C

Numbers in the figure represent the following concentrations of teleocidin added to Quin 2/DPPC vesicles: 0, 0 μM ; 1, 1.0 μM ; 2, 2.1 μM ; 3, 3.1 μM ; 4, 4.2 μM ; 5, 5.2 μM ; 6, 6.2 μM ; 7, 7.8 μM . Teleocidin was preincubated with Quin 2/DPPC vesicles, and then PLA_2 was added. [DPPC] = 0.14 mM, [PLA_2] = 71 nM and [Ca^{2+}] = 10 mM. The broken line shows the effect of the addition of teleocidin (4.2 μM) in the absence of PLA_2 .

PLA₂ activity (Fig. 2). At lower concentrations of teleocidin the fluorescence intensity of Quin 2 increased, but teleocidin at higher concentrations acted as an inhibitor of PLA₂. The addition of PMA or teleocidin alone to Quin 2/DPPC vesicles did not increase the fluorescence intensity of Quin 2 (Figs. 1 and 2). Therefore it appears that tumour promoters within a certain range of concentrations enhance markedly the hydrolytic activity of PLA₂.

Two possible mechanisms for the effects of tumour promoters on the hydrolytic activity of PLA₂ can be considered: (1) direct interaction of the tumour promoters with PLA₂, and (2) a change in the membrane structure due to the incorporation of the tumour promoter into the lipid membrane. In order to examine whether tumour promoters act directly on PLA₂ or on lipid membranes, the order of mixing of Quin 2/DPPC vesicles, tumour promoters and PLA₂ was changed.

Fig. 3. PMA activates PLA₂ by altering membrane structure

The broken lines show the results when PLA₂ was added to Quin 2/DPPC vesicles which had been preincubated with PMA; the solid lines indicate the results when PLA₂ was preincubated with PMA, followed by addition of Quin 2/DPPC vesicles. Numbers in the figure represent concentrations (μM) of PMA. Other experimental conditions were the same as those in Fig. 1.

Fig. 4. Teleocidin activates PLA₂ by altering membrane structure

Broken lines show the results when PLA₂ was added to Quin 2/DPPC vesicles which had been preincubated with teleocidin; solid lines indicate the results when PLA₂ was preincubated with teleocidin, followed by addition of Quin 2/DPPC vesicles. Numbers in the figure represent concentrations (μM) of teleocidin. Other experimental conditions were the same as those in Fig. 2.

The change in fluorescence intensity induced by PLA₂ which had been incubated previously with either 1.0 μM - or 3.1 μM -PMA but not with vesicles, did not differ much from that induced by PLA₂ alone (Fig. 3), indicating that PMA does not affect the intrinsic hydrolytic activity of PLA₂ upon binding to it. However, PMA at a concentration of 5.2 μM increased PLA₂ activity, which was however lower than that of PLA₂ when it was added to vesicles which had been preincubated with the same amount of PMA. The effect of teleocidin on PLA₂ activity was also dependent on the conditions of preincubation (Fig. 4). PLA₂ which was preincubated with this tumour promoter exhibited a prolonged latency period in the time course of the hydrolytic reaction once the vesicles were added as compared with PLA₂ added to Quin 2/DPPC vesicles previously incubated with tumour promoters.

These results indicate that tumour promoters adsorbed onto PLA₂ during preincubation in the absence of DPPC vesicles without affecting the hydrolytic activity of PLA₂, and were then slow to distribute to the lipid membrane upon mixing with DPPC vesicles. We conclude that the enhancement of PLA₂ activity by tumour promoters is caused by incorporation of the tumour promoters into the lipid bilayer membrane, inducing changes in the membrane structure, and not by the binding of tumour promoters to PLA₂.

This conclusion is consistent with the previous reports that PLA₂ hydrolyses membrane lipids by recognizing the structural irregularities of the membrane (Slotboom *et al.*, 1982, and references therein). A tightly packed lipid is not easily hydrolysed by PLA₂, because PLA₂ is not able to enter into the membrane (Thuren *et al.*, 1987; Van der Wiele *et al.*, 1988). As previously reported, these tumour promoters bind to the lipid bilayer membrane and cause changes in the membrane structure (Nam *et al.*, 1989a,b).

Fig. 5. Effect of tumour promoter on PLA₂ activity at various PLA₂ concentrations

PLA₂ was added to Quin 2/DPPC vesicles preincubated with teleocidin (2 μM) (□), PMA (2 μM) (○) or without tumour promoter (△). [DPPC] = 0.14 mM, [Ca²⁺] = 10 mM. The incubation time was 20 min.

Effects of varying the PLA₂ concentration

The effect of tumour promoters on PLA₂ activity was examined with various PLA₂ concentrations (Fig. 5). The extent of lipid hydrolysis by PLA₂ during the initial 20 min increased on raising the PLA₂ concentration. In the presence of PMA or teleocidin, the hydrolysis rate was enhanced and reached the plateau level above 100 nM-PLA₂. The saturation of hydrolysis by PLA₂ can be ascribed to the limiting number of possible binding sites of PLA₂ to the membrane, which are a result of distribution of the tumour promoter in the lipid membrane.

Distribution of PLA₂ to DPPC vesicles

The distribution of PLA₂ to DPPC vesicles was examined by measuring the fluorescence spectrum of a tryptophan residue in PLA₂. The third tryptophan residue is located in a hydrophobic

region of PLA₂ (Dijkstra *et al.*, 1981). When PLA₂ binds to lipid membrane, the emission of this tryptophan residue increases in intensity and shifts to the shorter wavelength. However, the fluorescence properties of PLA₂ did not change upon the addition of DPPC vesicles. These results are consistent with previous evidence that PLA₂ has a low affinity for DPPC vesicles (Menashe *et al.*, 1986). Neither PMA nor teleocidin induced significant changes in fluorescence intensity or wavelength of maximum emission within 5 min of PLA₂ addition.

Disturbances in membranes and PLA₂ activity by phorbol esters

Many derivatives of PMA have been synthesized and their activities as inflammatory and tumour-promoting agents studied. The effects of the phorbol esters PM, PDD and 4 α PDD on PLA₂ activity are shown in Fig. 6. PM affected PLA₂ activity similarly to PMA, whereas PDD and 4 α PDD had an insignificant effect on PLA₂ activity.

The ability of these phorbol esters to induce CF leakage from CF/DPPC vesicles was also evaluated (Fig. 7). CF leakage caused by PMA and PM was more significant than that due to PDD and 4 α PDD, indicating that monoacyl derivatives of phorbol disturb the membrane structure more markedly than do diacyl derivatives.

PMA and PM, which are monoacyl derivatives of phorbol, affected PLA₂ activity more than PDD and 4 α PDD. This result is also consistent with the interpretation that PLA₂ activity is strongly dependent on defects in the lipid membrane, because PMA and PM disturb the membrane structure markedly, whereas PDD and 4 α PDD have little effect. On the other hand, PMA and PDD are causative agents for inflammatory reactions and tumour promotion, but PM and 4 α PDD are inactive. Therefore the effects of phorbol esters on PLA₂ activity are not related to their potencies as inflammatory and tumour-promoting agents. Hitherto, two possibilities have been proposed for mediation of the effects of phorbol esters on phospholipase activity: a direct action on the phospholipase (Levine & Ohuchi, 1978; Halenda

Fig. 6. Time course of changes in fluorescence intensity of Quin 2 caused by PM (a), PDD (b) and 4 α PDD (c) with PLA₂ at 30 °C

Numbers in the figure represent the following concentrations of tumour promoter added to the Quin 2/DPPC vesicles: (a) 0, 0 μ M; 1, 1.0 μ M; 2, 3.1 μ M; 3, 5.2 μ M; 4, 7.8 μ M; 5, 10.4 μ M. (b) 0, 0 μ M; 1, 2.1 μ M; 2, 3.1 μ M; 3, 7.8 μ M. (c) 0, 0 μ M; 1, 2.1 μ M; 2, 3.1 μ M; 3, 5.2 μ M. Tumour promoter was preincubated with Quin 2/DPPC vesicles, and then PLA₂ was added. The broken lines indicate the results when phorbol ester (10.4 μ M) was added in the absence of PLA₂. [DPPC] = 0.14 mM; [PLA₂] = 71 nM; [Ca²⁺] = 10 mM. The results with PLA₂ alone differ from those under the same conditions in Figs. 1-4 due to the use of a different vesicle preparation.

Fig. 7. Effect of phorbol esters on CF leakage from DPPC vesicles at 30 °C

PM (1), PMA (2), 4 α PDD (3), PDD (4) and phorbol (5) were added to CF/DPPC vesicles. [DPPC] = 66 μ M; [phorbol ester] = 11 μ M.

et al., 1985), and via activation of protein kinase C, which phosphorylates a protein inhibitory towards phospholipase (Lytton & Mitchell, 1988; Halenda *et al.*, 1989). As far as pancreatic PLA₂ is concerned, the present study does not support the former mechanism for explaining the inflammatory reaction of phorbol esters.

It is worthwhile to note that an excess amount of a tumour promoter did not enhance PLA₂ activity. It is possible that PLA₂ is inactive in a lipid membrane which has many defects. The inability of PLA₂ to hydrolyse DPPC vesicles in the liquid crystalline phase is similarly explained (Menashe *et al.*, 1986). Tumour promoters within a certain range of concentrations might cause membrane irregularities which are thought to be involved in a reorganization of the enzyme-substrate complex or an enzyme-enzyme interaction.

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