Stimulation of prostaglandin E₂ synthesis by exogenous phospholipase A₂ and C in rabbit kidney medulla slices

Yohko FUJIMOTO, Naoki AKAMATSU, Atsuko HATTORI and Tadashi FUJITA
Department of Hygienic Chemistry, Osaka College of Pharmacy, Matsubara, Osaka 580, Japan

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We have investigated the effects of phospholipase A₂ and C on the synthesis of prostaglandin E₂ in rabbit kidney medulla and the release of fatty acids from the medulla slices. Exogenous phospholipase A₂ [from *Naja naja* (Indian cobra) venom] and phospholipase C (from *Clostridium welchii*) stimulated prostaglandin E₂ production in a dose-dependent manner. At the maximal effective concentrations (0.5 unit of phospholipase A₂/ml, 2 units of phospholipase C/ml), phospholipase C increased prostaglandin E₂ formation to the level observed with phospholipase A₂. Phospholipase A₂ enhanced the release only of unsaturated fatty acids, whereas phospholipase C stimulated the release of individual free fatty acids (C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2} and C_{20:4}). Moreover, *p*-bromophenacyl bromide inhibited phospholipase A₂-stimulated prostaglandin E₂ production and the release of fatty acids, but it had no influence on prostaglandin E₂ formation and the release of fatty acids increased by phospholipase C, indicating that the stimulatory effect of phospholipase C is not mediated through the activation of endogenous phospholipase A₂. These results suggest the presence of diacylglycerol lipase and monoacylglycerol lipase in the kidney and the importance of this pathway in prostaglandin synthesis by the kidney.

Several types of stimuli, including hormone stimulation (McGiff *et al.*, 1972; Needleman *et al.*, 1974; Schwartzman & Raz, 1979) or mechanical treatment (Piper & Vane, 1971; Palmer *et al.*, 1973), cause prostaglandin generation in kidney. The first step in the formation of prostaglandins is the release of precursor arachidonic acid from tissue lipids, since there are no significant amounts of preformed prostaglandins or free arachidonic acid in animal tissues. Phospholipase A₂ removes arachidonic acid from phospholipids, and a number of studies have suggested an important role for this enzyme in normal and pathological renal function (Limas & Limas, 1979; Baggio *et al.*, 1979; Morrison *et al.*, 1980).

However, it has been shown in platelets that the activation of phospholipase C precedes, and hence induces, the subsequent release of arachidonic acid (Bell *et al.*, 1979; Rittenhouse-Simmons, 1979; Lapetina & Cuatrecasas, 1979; Billah *et al.*, 1979). Although these investigators have reported the importance of phospholipase C pathway in prostaglandin synthesis by platelets, the importance of this pathway in other cells is yet to be determined. Recently, the presence of phospholipase C activity in kidney has been shown (Lipsky & Lietman,

1982). It has also been reported that exogenously added phospholipase A₂ [from porcine pancreas and *Naja naja* (Indian cobra) venom] and phospholipase C (from the anaerobic bacterium *Clostridium welchii*) can mimic the action of phospholipase A₂ and C stimulators which activate endogenous membrane-located phospholipase A₂ and C in several types of cells (Verkleij *et al.*, 1973; Flynn *et al.*, 1981; Nemezc *et al.*, 1981). From this viewpoint, treatment of kidney medulla with phospholipase A₂ and C might offer a means of stimulating biochemical events leading to the release of arachidonic acid from membrane phospholipids. Therefore we investigated the effects of exogenous phospholipase A₂ and phospholipase C on the synthesis of prostaglandin E₂ in kidney medulla and the release of fatty acids from the medulla slices.

Materials and methods

Materials

Phospholipase A₂ of *Naja naja* venom (700 units/mg of protein), phospholipase C of *Clostridium welchii* (425 units/mg of protein, Type XII), phosphatidylcholine (egg yolk) and mepacrine

(quinacrine) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *p*-Bromophenacyl bromide was obtained from Wako Pure Chemical Industries, Osaka, Japan. All other reagents were analytical grade.

Methods

Tissue. Male rabbits (2–2.5 kg) were used in the present study. The kidneys were removed from anaesthetized (sodium pentobarbital, 30 mg/kg) rabbit and rapidly chilled in ice-cold 0.9% NaCl. The kidney medulla slices were prepared as described elsewhere (Fujimoto & Fujita, 1982).

Incubation of medulla slices. In all experiments, rabbit kidney medulla slices (0.4 g) were pre-incubated in 4.0 ml of 0.15 M-KCl/0.02 M-Tris/HCl buffer, pH 7.4, at 4°C for 5 min. After pre-incubation, the medium was changed to a new solution containing the indicated concentrations of enzymes. Incubation was continued for another 30 min or 60 min to measure prostaglandin E₂ and free (non-esterified)-fatty-acid release.

Determination of prostaglandin formation. After incubation the medium was assayed for prostaglandin E₂ content by a high-pressure liquid-chromatographic method (Fujimoto *et al.*, 1983). Briefly, prostaglandin E₂ extracted with ethyl acetate (approx. pH 3) was measured after its base-catalysed conversion to prostaglandin B₂ (Jouvenaz *et al.*, 1970). Peak heights were measured for the quantification of the extracted prostaglandin B₂ relative to a prostaglandin B₂ standard prepared from authentic prostaglandin E₂.

Fatty acid analysis. The individual free fatty acids in the incubation medium were measured as described previously (Yasuda *et al.*, 1980). To separate the lipid fractions (free fatty acids, triacylglycerols etc.), the total lipids extracted with chloroform/methanol (2:1, v/v) were applied on the thin-layer plate (silica-gel H; 0.5 mm thick; Merck) under a stream of N₂ and then developed with light petroleum (b.p. 30–70°C)/diethyl ether/acetic acid (80:30:1, by vol.). The free-fatty-acid zone was extracted, and methyl esters were prepared and quantitatively determined by g.l.c. The concentration of the individual fatty acids in the medium was evaluated from the peak area calculated by the triangulation, in comparison with the known amounts of margaric acid (15 µg), which appeared as a new peak.

Use of egg-yolk phosphatidylcholine liposomes. To determine the effects of *p*-bromophenacyl bromide and mepacrine on phospholipase A₂ activity, a liposomal suspension of egg-yolk phosphatidylcholine was used as the substrate for phospholipase A₂ from *Naja naja* venom. The enzyme activity was measured as the amount of fatty acid released from the substrate in the reaction mixture (Yasuda

& Fujita, 1977). Phosphatidylcholine liposomes were prepared as follows. Solutions of phosphatidylcholine in chloroform were evaporated to dryness *in vacuo*. Residual lipid was dispersed in 0.15 M-KCl/0.02 M-Tris/HCl buffer by shaking with some glass beads at approx. 35°C. Phospholipase A₂ was preincubated with *p*-bromophenacyl bromide or mepacrine in the Tris/HCl buffer for 10 min at 37°C before use. Incubations were carried out at 37°C in the Tris/HCl buffer at a lipid concentration of 1 mg/ml in a volume of 2.0 ml. CaCl₂ was added to a final concentration of 10 mM. The reaction was initiated by the addition of 3 units of phospholipase A₂ and terminated after 30 min by the addition of 20 ml of chloroform/methanol (2:1, v/v). The total lipids were separated by t.l.c. and the fraction containing free fatty acids was extracted and quantitatively measured by g.l.c. (Yasuda *et al.*, 1980).

Statistics. Results are means ± s.e.m. Statistical significance was determined by Student's *t* test.

Results

Fig. 1 illustrates the effects of various amounts of phospholipase A₂ of *Naja naja* venom and phospholipase C of *Clostridium welchii* on prostaglandin E₂ synthesis in rabbit kidney medulla slices. Exogenous phospholipase A₂ and phospholipase C showed dose-dependent stimulation of prostaglandin E₂ production. The minimally effective stimulatory concentration of phospholipase A₂ and phospholipase C was 0.1 unit/ml (2-fold) and 0.5 unit/ml (1.4-fold) respectively. Maximal

Fig. 1. Effects of phospholipase A₂ and phospholipase C on prostaglandin E₂ synthesis of rabbit kidney medulla slices. Slices were incubated for 30 min at 37°C in 0.15 M-KCl/0.02 M-Tris/HCl buffer in the presence of different concentrations of phospholipase A₂ (●) or phospholipase C (▲). Each point represents the mean result for three experiments.

effect on prostaglandin E_2 production was observed with 0.5 unit of phospholipase A_2 /ml or 2 units of phospholipase C/ml. In addition, 2 units of phospholipase C/ml stimulated medullary generation of prostaglandin E_2 to the same extent as 0.5 unit of phospholipase A_2 /ml. No further stimulation was noted when concentrations of these enzymes were raised to 5 units/ml. Neither phospholipase A_2 nor phospholipase C had a toxic effect on prostaglandin E_2 synthesis, even at the highest concentration. The concentrations of 0.5 unit of phospholipase A_2 /ml and 2 units of phospholipase C/ml were selected in the further experiments.

The time course of these enzyme-induced increases in prostaglandin E_2 release from rabbit kidney medulla slices is shown in Fig. 2. Addition of phospholipase A_2 (0.5 unit/ml) produced a rapid increase in the release of prostaglandin E_2 , with no apparent latency; this increase proceeded in a linear manner for 60 min. Phospholipase C (2 units/ml) stimulated the release of prostaglandin E_2 after a lag period of about 10 min, but by 30 min the release of prostaglandin E_2 was increased to a

Fig. 2. Time course of phospholipase A_2 - and phospholipase C-induced increases in prostaglandin E_2 release from rabbit kidney medulla slices

Slices were incubated for 60 min at 37°C in 0.15M-KCl/0.02M-Tris/HCl buffer. Each point represents the mean result for three experiments. ○, Control; ●, phospholipase A_2 (0.5 unit/ml); ▲, phospholipase C (2 units/ml).

level comparable with that obtained with phospholipase A_2 .

p-Bromophenacyl bromide and mepacrine have been shown to inhibit the release of arachidonic acid and the generation of arachidonate-oxygenated products from blood platelets (Vallee *et al.*, 1979). Recently we have reported that mepacrine at a concentration of 1.6 mM inhibits medullary generation of prostaglandin E_2 by 70% (Fujimoto *et al.*, 1983). As shown in Table 1, *p*-bromophenacyl bromide (0.1 mM) and mepacrine (1.6 mM) inhibited phospholipase A_2 -stimulated prostaglandin E_2 production by 58 and 48% respectively. We found that at lower concentrations these effects were not statistically significant (results not shown). On the other hand, *p*-bromophenacyl bromide (0.1 mM) failed to inhibit phospholipase C-stimulated prostaglandin E_2 formation, whereas mepacrine (1.6 mM) inhibited it (43%). *p*-Bromophenacyl bromide has been found to inhibit the activity of phospholipase A_2 from porcine pancreas and snake venom directly (Volwerk *et al.*, 1974; Vallee *et al.*, 1979), apparently through interaction with specific amino acid residue(s) in the enzyme (Volwerk *et al.*, 1974). Dise *et al.* (1982) reported that mepacrine interacted directly with membrane phospholipids, primarily phosphatidylethanolamine, to form less-polar derivatives, and that mepacrine-phospholipid interaction could alter membrane architecture and disrupt membrane protein-lipid inter-

Table 1. Effects of *p*-bromophenacyl bromide and mepacrine on phospholipase A_2 - or phospholipase C-induced prostaglandin E_2 release from rabbit kidney medulla slices

Slices were incubated for 30 min at 37°C in 0.15M-KCl/0.02M-Tris/HCl buffer. Values are means $\pm$ S.E.M. ($n = 5$). * indicates significantly different ($P < 0.01$) from the value observed with phospholipase A_2 (Expt. 1) or phospholipase C (Expt. 2).

	Prostaglandin E_2 (μ g/g wet wt. of tissue)
Expt. 1	
None	2.52 $\pm$ 0.12
Phospholipase A_2 (0.5 unit/ml)	11.02 $\pm$ 1.21
Phospholipase A_2 + <i>p</i> -bromophenacyl bromide (0.1 mM)	4.59 $\pm$ 0.53*
Phospholipase A_2 + mepacrine (1.6 mM)	5.68 $\pm$ 0.69*
Expt. 2	
None	2.30 $\pm$ 0.09
Phospholipase C (2 units/ml)	9.50 $\pm$ 0.43
Phospholipase C + <i>p</i> -bromophenacyl bromide (0.1 mM)	10.11 $\pm$ 0.34
Phospholipase C + mepacrine (1.6 mM)	5.43 $\pm$ 0.69*

actions. Thus it is possible to ascribe the inhibitory effect of mepacrine towards phospholipases A₂ and C to a substrate-inhibitor interaction.

To clarify this point, we examined the effects of *p*-bromophenacyl bromide and mepacrine on phospholipase A₂ activity by using a liposomal suspension of phosphatidylcholine as the substrate (Table 2). Controls, incubated in the absence of phospholipase, did not show any detectable lipid degradation. Addition of phospholipase A₂ (3 units) caused the liberation of only unsaturated fatty acids (oleic acid, linoleic acid and arachidonic acid) from the substrate. When phospholipase A₂ was pretreated with *p*-bromophenacyl bromide and mepacrine, *p*-bromophenacyl bromide (0.01–0.1 mM) inactivated this enzyme in a dose-dependent manner, whereas mepacrine (0.8–3.2 mM) had no effect on the activity. This result strongly suggests that *p*-bromophenacyl bromide is a direct inhibitor of phospholipase A₂ and that mepacrine might work in an indirect way.

In contrast with above observations, Vigo *et al.* (1980) have reported that mepacrine inhibits phospholipase A₂ by a direct interaction with the enzyme. It must be pointed out, however, that studies utilizing mepacrine should be interpreted with caution unless adequate control experiments, excluding direct mepacrine-phospholipid derivatives (Dise *et al.*, 1982), are performed. They measured phospholipase A₂ activity as the amount of lysophospholipid instead of fatty acid released, but did not describe the formation of mepacrine-lysophospholipid derivatives. Detection of such derivatives would require measurements of phospholipid distribution by t.l.c. in a basic system. Such measurements were not carried out in their study with mepacrine.

Furthermore, we determined the effects of phospholipase A₂ and phospholipase C on the release of fatty acids from kidney medulla slices (Table 3). Medulla slices were incubated for 30 min at 37°C with phospholipase A₂ and phospholipase

Table 2. Effects of *p*-bromophenacyl bromide and mepacrine on phospholipase A₂ activity using egg-yolk phosphatidylcholine liposomes

Phospholipase A₂ was pretreated with *p*-bromophenacyl bromide or mepacrine in 0.15M-KCl/0.02M-Tris/HCl buffer for 10 min at 37°C before use. Incubations were carried out for 30 min at 37°C in the Tris/HCl buffer containing 10 mM-CaCl₂ in a total volume of 2.0 ml. The final concentration of phosphatidylcholine was 1 mg/ml. '+' indicates a trace amount. For further details, see the Materials and methods section. Values are means ± S.E.M. (n = 5).

	Free fatty acids released from the substrate (μg)		
	C _{18:1}	C _{18:2}	C _{20:4}
Phospholipase A ₂ (3 units)	23.42 ± 1.83	13.42 ± 1.20	0.78 ± 0.08
Plus <i>p</i> -bromophenacyl bromide			
0.01 mM	18.57 ± 0.26	11.04 ± 0.44	0.63 ± 0.05
0.05 mM	13.42 ± 0.50	8.50 ± 0.33	0.20 ± 0.01
0.1 mM	9.47 ± 0.20	5.82 ± 0.48	+
Plus mepacrine			
0.8 mM	23.36 ± 0.25	12.51 ± 0.14	0.73 ± 0.04
1.6 mM	20.92 ± 0.24	12.09 ± 0.40	0.66 ± 0.05
3.2 mM	20.10 ± 0.34	11.50 ± 0.71	0.65 ± 0.06

Table 3. Effects of phospholipase A₂ and phospholipase C on the release of fatty acids from rabbit kidney medulla slices. Slices were incubated for 30 min at 37°C in 0.15M-KCl/0.02M-Tris/HCl buffer. Values are means ± S.E.M. (n = 3).

	Fatty acids released (μg/g wet wt. of tissue)				
	C _{16:0}	C _{18:0}	C _{18:1}	C _{18:2}	C _{20:4}
None	16.01 ± 1.21	5.75 ± 0.32	4.56 ± 0.32	1.55 ± 0.13	0.13 ± 0.01
Phospholipase A ₂ (0.5 unit/ml)	15.72 ± 1.05	5.84 ± 0.43	12.09 ± 1.15	11.41 ± 1.52	2.28 ± 0.15
Phospholipase A ₂ + <i>p</i> -bromophenacyl bromide (0.1 mM)	15.23 ± 0.95	6.44 ± 0.72	2.21 ± 0.19	2.01 ± 0.17	0.08 ± 0.01
Phospholipase A ₂ + mepacrine (1.6 mM)	16.75 ± 1.53	6.37 ± 0.53	3.38 ± 0.28	2.77 ± 0.32	0.09 ± 0.01
Phospholipase C (2 units/ml)	27.22 ± 1.32	11.05 ± 0.86	8.90 ± 0.81	9.07 ± 0.65	0.90 ± 0.05
Phospholipase C + <i>p</i> -bromophenacyl bromide (0.1 mM)	26.56 ± 1.45	10.28 ± 0.51	9.38 ± 0.89	9.37 ± 0.38	0.91 ± 0.07
Phospholipase C + mepacrine (1.6 mM)	17.37 ± 1.44	6.69 ± 0.43	6.02 ± 0.55	4.72 ± 0.52	0.46 ± 0.02

C in 0.15M-KCl/0.02M-Tris/HCl buffer. Phospholipase A₂ is an enzyme that hydrolyses the ester linkage in the 2-position of phosphoacylglycerol. In our study, phospholipase A₂ stimulated the release of unsaturated fatty acids such as oleic acid, linoleic acid and arachidonic acid. The effect of phospholipase A₂ could be blocked by the addition of *p*-bromophenacyl bromide (0.1 mM) and mepacrine (1.6 mM). These results imply that the decreased lipolysis and the resulting decreased availability of free arachidonic acid is the main cause for the inhibition of phospholipase A₂-stimulated prostaglandin E₂ formation by *p*-bromophenacyl bromide and mepacrine.

On the other hand, phospholipase C enhanced the release of individual free fatty acids (C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2} and C_{20:4}) from kidney medulla slices (Table 3). The effect of phospholipase C was not inhibited by *p*-bromophenacyl bromide (0.1 mM), but was inhibited by mepacrine (1.6 mM). This result is consistent with the effect of these agents on phospholipase C-stimulated prostaglandin E₂ production.

Discussion

Intracellular arachidonic acid levels control prostaglandin synthesis. Ultimately the availability of free arachidonic acid to prostaglandin cyclo-oxygenase rather than its total concentration must determine the synthesis of prostaglandins; hence, knowledge of the factors that regulate the levels of free arachidonic acid is essential for an understanding of the control of prostaglandin production. As shown in Fig. 1, exogenously applied phospholipase C stimulated medullary generation of prostaglandin E₂ to a level comparable with that obtained with exogenous phospholipase A₂. This result suggests the possibility that phospholipase C pathway is important for prostaglandin synthesis in the kidney.

The stimulatory effect of phospholipase C on prostaglandin E₂ synthesis could be achieved by the activation of endogenous phospholipase A₂, or through the involvement of a diacylglycerol lipase activity. As a consequence of phospholipase C stimulation, there is a transient accumulation of 1,2-diacylglycerol (Bell *et al.*, 1979; Rittenhouse-Simmons, 1979), which is followed by its disappearance, due to effective phosphorylation of 1,2-diacylglycerol to phosphatidic acid by diacylglycerol kinase (Billah *et al.*, 1979) and/or to hydrolysis by diacylglycerol lipase (Bell *et al.*, 1979). Diacylglycerol is converted into free fatty acid and 2-arachidonylmonoacylglycerol by diacylglycerol lipase and then to glycerol plus free arachidonic acid by monoacylglycerol lipase (Chau & Tai, 1981). On the other hand, the formation of phos-

phatidic acid could initiate mobilization of Ca²⁺ (Tyson *et al.*, 1976; Gerrard *et al.*, 1978), which in turn could stimulate phospholipase A₂ activity (Rittenhouse-Simmons & Deykin, 1978; Lapetina *et al.*, 1980). In addition, Lapetina *et al.* (1980) showed a possible role of phosphatidic acid in a transesterification mechanism in which arachidonic acid is transferred from phosphatidic acid to other phospholipids.

The present study showed that *p*-bromophenacyl bromide inhibited phospholipase A₂-stimulated prostaglandin E₂ production and the release of fatty acids, but that it had no influence on prostaglandin E₂ formation and the release of fatty acids increased by phospholipase C (Tables 1 and 3). It is possible that phospholipase C of *Clostridium welchii* is stable in the presence of *p*-bromophenacyl bromide. Recent studies have demonstrated that *p*-bromophenacyl bromide can inhibit phosphatidylinositol-specific phospholipase C from platelets, possibly by interaction with thiol groups (Hofmann *et al.*, 1982). A similar tendency was observed after treatment with *p*-chloromercuribenzoic acid, which reacts preferentially with thiol groups; phospholipase C of *Bacillus cereus* was not inhibited by *p*-chloromercuribenzoic acid, but phosphatidylinositol-specific phospholipases C of guinea-pig brain and intestinal mucosa were blocked by *p*-chloromercuribenzoic acid (Brockerhoff & Jensen, 1974). However, *p*-bromophenacyl bromide, at least, inhibits endogenous phospholipase A₂, which is structurally very similar to snake-venom phospholipase A₂ (Van den Bosch, 1980). Thus it seems likely that the enhancement of prostaglandin E₂ formation elicited by phospholipase C can be ascribed to increased availability of free arachidonic acid by diacylglycerol lipase and monoacylglycerol lipase, rather than by way of phosphatidic acid as an intermediate, which in turn suggests the presence of diacylglycerol lipase and monoacylglycerol lipase in the kidney. This is reinforced by the fact that phospholipase C stimulates the release of individual free fatty acids (C_{16:0}, C_{18:0}, C_{18:1}, C_{18:2} and C_{20:4}), whereas phospholipase A₂ enhances the release of only unsaturated fatty acids from kidney medulla slices (Table 3).

Arachidonic acid released from phospholipids is a substrate for prostaglandin formation, and prostaglandin generation under physiological conditions must be preceded by a lipolytic process to release free arachidonic acid from tissue lipids. The present results suggest the presence of diacylglycerol lipase and monoacylglycerol lipase in the kidney and the importance of this pathway in prostaglandin synthesis by the kidney.

It has been reported that kinins, angiotensin and catecholamines cause prostaglandin generation in

kidney (McGiff *et al.*, 1972; Needleman *et al.*, 1974; Schwartzman & Raz, 1979). The possible mechanism of action of these hormones which may affect phospholipase C activity in the kidney requires further investigation and might open new possibilities in the further understanding of the mechanisms of arachidonic acid release for prostaglandin synthesis in the kidney.

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