Effect of diarachidonin on prostaglandin E₂ synthesis in rabbit kidney medulla slices

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The effect of diarachidonin on the synthesis of prostaglandin E₂ in rabbit kidney medulla slices was examined. The addition of diarachidonin stimulated prostaglandin E₂ production in a dose-dependent manner. At three concentrations (10, 50 and 100 μ M), increases in prostaglandin E₂ formation induced by exogenous diarachidonin were 2-fold greater than those induced by exogenous arachidonic acid. Diacylglycerol or phosphatidic acid from egg lecithin had little or no effect on prostaglandin E₂ production. Moreover, EGTA failed to inhibit diarachidonin-stimulated prostaglandin E₂ formation, indicating that the stimulatory effect of diarachidonin is not mediated through the activation of endogenous phospholipase A₂ (including phosphatidic acid-specific phospholipase A₂). These results are discussed in the light of our former hypothesis that arachidonic acid release from kidney medulla phospholipids might occur through the sequential action of a phospholipase C coupled to diacylglycerol and monoacylglycerol lipases [Fujimoto, Akamatsu, Hattori & Fujita (1984) *Biochem. J.* **218**, 69–74].

INTRODUCTION

The rate-limiting step in the synthesis of prostaglandins is the mobilization of arachidonic acid from tissue lipid stores (Vogt, 1978; Isakson *et al.*, 1978). At least three enzymic pathways have been proposed to mediate release of arachidonic acid for prostaglandin synthesis in kidney medulla and other tissues: phosphatidylinositol-specific phospholipase C, phospholipase A₂ and triacylglycerol lipase (Walenga *et al.*, 1981; Bell *et al.*, 1979; Tobian & O'Donnell, 1976; Michell, 1975). Since phosphatidylinositol of mammalian tissues is almost exclusively in the 1-stearoyl-2-arachidonyl form, the phosphatidylinositol-specific phospholipase C pathway allows for the specific release of arachidonic acid (Bell *et al.*, 1979). Recently, we have reported that exogenously applied phospholipase C stimulates medullary generation of prostaglandin E₂ to a level comparable with that obtained with exogenous phospholipase A₂ (Fujimoto *et al.*, 1984). This study has suggested the possibility that phospholipase C–diacylglycerol lipase pathway is important for prostaglandin synthesis in the kidney.

Following formation of diacylglycerol by phospholipase C, arachidonic acid is either released by diacylglycerol and monoacylglycerol lipases, or the diacylglycerol is phosphorylated to phosphatidic acid (Bell *et al.*, 1979; Billah *et al.*, 1979). Arachidonic acid may then be released from phosphatidic acid by the action of phosphatidic acid-specific phospholipase A₂ (Billah *et al.*, 1981). While it is clear that activation of phospholipase C yields diacylglycerol, the fate of the arachidonic acid which enters the diacylglycerol pool and the pathway to its liberation in the kidney have remained obscure. The present paper reports about the effect of diarachidonin on the biosynthesis of prostaglandin E₂ in kidney medulla slices. Our data are discussed in view of the possible role of diacylglycerol and monoacylglycerol lipases in providing arachidonic acid to cyclo-oxygenase in kidney medulla.

MATERIALS AND METHODS

Materials

Diarachidonin was obtained from Nu-Chek Prep, Inc. (Elysian MN, U.S.A.), and diacylglycerol from egg lecithin was obtained from Serdary Research Laboratories, London, Ontario, Canada. Phosphatidic acid from egg lecithin, arachidonic acid and mepacrine (quinacrine) were obtained from Sigma. *p*-Bromophenacyl bromide and EGTA were purchased 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) rabbits and rapidly chilled in ice-cold 0.9% NaCl. 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 preincubated 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 preincubation, the medium was changed to a new solution containing the indicated concentrations of diarachidonin, arachidonic acid, diacylglycerol or phosphatidic acid. Each lipid was firstly dissolved in dimethyl sulphoxide and then diluted 100-fold into the reaction mixture. Dimethyl sulphoxide at 1% (v/v) had no effect on prostaglandin E₂ production in medulla slices. Incubation was continued for another 30 min to measure prostaglandin E₂. In experiments utilizing EGTA, slices were preincubated for 30 min at 37 °C in the Tris/HCl buffer with or without 2 mM-EGTA. At *t* = 30 min, slices were washed briefly and transferred to fresh buffer with or without 2 mM-EGTA, and the incubation was continued to *t* = 60 min to measure

Fig. 1. Effects of exogenous arachidonic acid and diarachidonin on prostaglandin E_2 release from 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 arachidonic acid (●) or diarachidonin (▲). Each point represents the mean for four experiments (S.E.M. values were less than 5%). The mean value for prostaglandin E_2 release at zero arachidonic acid or diarachidonin (3.45 $\mu\text{g/g}$ wet wt. of tissue) was subtracted from the corresponding values at the different arachidonic acid or diarachidonin concentrations and the obtained differences, which represent net arachidonic acid- or diarachidonin-stimulated prostaglandin E_2 release, were plotted.

prostaglandin E_2 . Diarachidonin was added at $t = 30$ min to the incubations.

Determination of prostaglandin formation. After incubation the medium was assayed for prostaglandin E_2 content by a h.p.l.c. method (Fujimoto *et al.*, 1983). Briefly, prostaglandin E_2 extracted with ethyl acetate (pH approx. 3) was measured after its base-catalysed conversion to prostaglandin B_2 (Jouvenaz *et al.*, 1970). Peak heights were measured for the quantification of the extracted prostaglandin B_2 relative to a prostaglandin B_2 standard prepared from authentic prostaglandin E_2 .

Statistics. Results are means $\pm$ S.E.M. Statistical significance was determined by Student's t test.

RESULTS AND DISCUSSION

Fig. 1 illustrates the effects of various concentrations of arachidonic acid and diarachidonin on prostaglandin E_2 synthesis in rabbit kidney medulla slices. Added exogenous arachidonic acid and diarachidonin showed dose-dependent stimulation of prostaglandin E_2 production. At three concentrations (10, 50 and 100 μM),

Table 1. Effects of diacylglycerol and phosphatidic acid from egg lecithin on prostaglandin E_2 release from rabbit kidney medulla slices

Slices were incubated for 30 min at 37 °C in 0.15 M-KCl/0.02 M-Tris/HCl buffer. Values are means $\pm$ S.E.M. ($n = 3$). * Indicates significantly different ($P < 0.01$) from control.

Treatment	Prostaglandin E_2 ($\mu\text{g/g}$ wet wt. of tissue)
Control	3.48 $\pm$ 0.03
Diarachidonin (50 μM , 33.3 $\mu\text{g/ml}$)	5.61 $\pm$ 0.13*
Diacylglycerol	
33.3 $\mu\text{g/ml}$	3.57 $\pm$ 0.10
66.6 $\mu\text{g/ml}$	3.64 $\pm$ 0.13
Phosphatidic acid	
33.3 $\mu\text{g/ml}$	3.50 $\pm$ 0.09
66.6 $\mu\text{g/ml}$	3.56 $\pm$ 0.08

diarachidonin increased prostaglandin E_2 release approx. 2-fold compared with the value observed with arachidonic acid.

Dawson *et al.* (1983) and Hofmann & Majerus (1982) demonstrated that 1,2-diacylglycerols potentiate the ability of phospholipases A_2 and C to hydrolyse phospholipids in cell-free systems and microsomes. It was suggested that this effect may result from 1,2-diacylglycerol disruption of normal bilayer structure. On the other hand, the formation of phosphatidic acid from diacylglycerol by diacylglycerol kinase (Bell *et al.*, 1979; Billah *et al.*, 1979) could initiate mobilization of Ca^{2+} (Tyson *et al.*, 1976; Gerrard *et al.*, 1978), which in turn could stimulate phospholipase A_2 activity (Rittenhouse-Simmons & Deykin, 1978; Lapetina *et al.*, 1980).

In order to ascertain these possibilities, we examined the effects of diacylglycerol and phosphatidic acid from egg lecithin on the release of prostaglandin E_2 from rabbit kidney medulla slices (Table 1). In preliminary experiments, the percentage composition of fatty acids of diacylglycerol or phosphatidic acid from egg lecithin was determined by g.l.c. (Yasuda *et al.*, 1980). The major fatty acid of diacylglycerol or phosphatidic acid was palmitic acid (43%) and arachidonic acid was present in a minor percentage (1.5%). Diarachidonin significantly increased prostaglandin E_2 release at 50 μM (33.3 $\mu\text{g/ml}$) compared with the control. By contrast, when tested at concentrations up to 66.6 $\mu\text{g/ml}$, diacylglycerol or phosphatidic acid from egg lecithin had little or no effect on prostaglandin E_2 release. These results do not support a possible role for the structure of diacylglycerol or phosphatidic acid in mediating the release of arachidonic acid for prostaglandin synthesis. Similar results were observed by Watson *et al.* (1984) who showed that 1,2-diacylglycerols did not potentiate the action of phospholipases A_2 and C in human platelets. The failure to find significant stimulation of prostaglandin E_2 generation using diacylglycerol or phosphatidic acid from egg lecithin may result from the fact that the total concentration of arachidonic acid in such lipids is insufficient for prostaglandin E_2 synthesis in kidney medulla slices as compared with diarachidonin. It can be conceived that degradation of diarachidonin itself is the main cause for diarachidonin-stimulated prostaglandin E_2 formation.

Table 2. Effects of EGTA on diarachidonin-induced prostaglandin E₂ release from rabbit kidney medulla slices

Slices were preincubated for 30 min at 37 °C in 0.15 M-KCl/0.02 M-Tris/HCl buffer with or without 2 mM-EGTA. At $t = 30$ min, slices were washed briefly and transferred to fresh buffer with or without 2 mM-EGTA, and the incubation was continued to $t = 60$ min to measure prostaglandin E₂. Diarachidonin, 50 μ M, was added at $t = 30$ min to the incubations. Values are means $\pm$ S.E.M. ($n = 4$). Effects of EGTA on diarachidonin-induced prostaglandin E₂ release were determined as the difference in the amounts of prostaglandin E₂ released in the presence and absence of diarachidonin.

Treatment	Prostaglandin E ₂ (μ g/g wet wt. of tissue)		
	No diarachidonin	+ 50 μ M-diarachidonin	Difference
Control	2.87 $\pm$ 0.15	4.30 $\pm$ 0.38	1.43
+ EGTA (2 mM)	1.29 $\pm$ 0.05	2.79 $\pm$ 0.16	1.50

Table 3. Effects of mepacrine and *p*-bromophenacyl bromide on diarachidonin-induced prostaglandin E₂ release from rabbit kidney medulla slices

Slices were incubated for 30 min at 37 °C in 0.15 M-KCl/0.02 M-Tris/HCl buffer. Values are means $\pm$ S.E.M. ($n = 5$). Effects of mepacrine and *p*-bromophenacyl bromide on diarachidonin-induced prostaglandin E₂ release were determined as the difference in the amounts of prostaglandin E₂ released in the presence and absence of diarachidonin.

Treatment	Prostaglandin E ₂ (μ g/g wet wt. of tissue)		
	No diarachidonin	+ 50 μ M-diarachidonin	Difference
Control	3.45 $\pm$ 0.08	5.58 $\pm$ 0.24	2.13
Mepacrine (1.6 mM)	1.04 $\pm$ 0.02	1.90 $\pm$ 0.20	0.86
<i>p</i> -Bromophenacyl bromide (0.1 mM)	2.26 $\pm$ 0.24	4.45 $\pm$ 0.31	2.19

The stimulatory effect of diarachidonin on prostaglandin E₂ synthesis could be achieved by the activation of endogenous diacylglycerol and monoacylglycerol lipases, or through the involvement of a phosphatidic acid-specific phospholipase A₂ activity. Billah *et al.* (1981) reported that a phospholipase A₂ activity specific for phosphatidic acid was dependent on Ca²⁺ and this activity was completely inhibited by EGTA. Therefore, we determined the effect of EGTA on diarachidonin-induced prostaglandin E₂ release from rabbit kidney medulla slices (Table 2). EGTA reduced the production of basal prostaglandin E₂ by 55% at a concentration of 2 mM. It has been shown that phospholipase C activation does not depend on cytoplasmic increases of calcium concentration, at variance with phospholipase A₂ (Simon *et al.*, 1984; Rittenhouse, 1984). The inhibition by EGTA of basal prostaglandin E₂ formation appears to be mediated via inhibition of a Ca²⁺-dependent phospholipase A₂ that cleaves arachidonic acid from phospholipids, primarily phosphatidylcholine. In the present study, EGTA had no influence on diarachidonin-stimulated prostaglandin E₂ formation. Thus it seems likely that the enhancement of prostaglandin E₂ formation elicited by diarachidonin can be ascribed to increased availability of free arachidonic acid by diacylglycerol lipase and monoacylglycerol lipase, rather than by way of a phosphatidic acid-specific phospholipase A₂, which in turn suggests the presence of diacylglycerol lipase and monoacylglycerol lipase in the kidney. This is reinforced by the fact that the addition of diarachidonin produced a 2-fold greater increase in

prostaglandin E₂ release than was seen in response to arachidonic acid (Fig. 1). The failure of EGTA to inhibit diarachidonin-stimulated prostaglandin E₂ formation also suggests that diacylglycerol and monoacylglycerol lipases of kidney medulla are independent of Ca²⁺. This result is in agreement with previous data (Chau & Tai, 1981) which indicated that there was no effect of EDTA or Ca²⁺ on human platelet diacylglycerol or monoacylglycerol lipase activity. Authi *et al.* (1985) also demonstrated that diacylglycerol lipase in human platelet intracellular and surface membranes was optimally active in the absence of calcium (stimulated by 1 mM-EGTA and partially inhibited by added calcium). In the same study they suggested that the phospholipase C-diacylglycerol lipase pathway might be independent of changes in cell calcium levels.

Mepacrine and *p*-bromophenacyl bromide 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 reported that mepacrine at a concentration of 1.6 mM, and *p*-bromophenacyl bromide at 0.1 mM, inhibited exogenous phospholipase A₂-stimulated prostaglandin E₂ production by 48% and 58% respectively (Fujimoto *et al.*, 1984). In the same study, we suggested that *p*-bromophenacyl bromide was a direct inhibitor of phospholipase A₂ and that mepacrine might work in an indirect way. As shown in Table 3, diarachidonin-induced prostaglandin E₂ release of 2.13 μ g/g of tissue was reduced by treatment with mepacrine (1.6 mM) to 0.86 μ g/g of tissue. In

contrast, *p*-bromophenacyl bromide (0.1 mM) failed to inhibit diarachidonin-stimulated prostaglandin E_2 formation.

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 interactions. It is possible to ascribe the inhibitory effect of mepacrine towards diacylglycerol and monoacylglycerol lipases to a phospholipid-mepacrine interaction. On the other hand, it has been shown that *p*-bromophenacyl bromide can inhibit phosphatidylinositol-specific phospholipase C from platelets, possibly by interaction with thiol groups (Hofmann *et al.*, 1982; Kyger & Franson, 1984). The lack of effect of *p*-bromophenacyl bromide on diarachidonin-induced prostaglandin E_2 release was interpreted as indicating that sulphhydryl groups were not essential for the activation of diacylglycerol and monoacylglycerol lipases in kidney medulla. Similar properties have been described for rat plasma and platelet monoacylglycerol lipases (Fielding, 1981).

The results of this work suggest the presence of diacylglycerol lipase and monoacylglycerol lipase in the kidney and the importance of this pathway in prostaglandin synthesis by the kidney. Also, a study of some properties of these enzymes was performed. However, it does not necessarily support that the diacylglycerol/monoacylglycerol lipases pathway plays a major role in arachidonic acid release for prostaglandin synthesis in the kidney. Direct deacylation of phospholipids by phospholipase A_2 has been recognized as a favourable pathway, since this represents the simplest mode of release of arachidonic acid (Rittenhouse-Simmons *et al.*, 1976; McKean *et al.*, 1981). In renal medulla, triacylglycerol lipase activity is also a potential mediator of release of arachidonic acid for prostaglandin synthesis (Bojesen, 1969, 1974; Bohman & Jensen, 1976; Tobian & O'Donnell, 1976). The contribution by the diacylglycerol/monoacylglycerol lipases pathway to overall arachidonic acid release for prostaglandin synthesis in the kidney still remains to be determined.

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