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Diacylglycerol stimulates phospholipase A_2 from Swiss 3T3 fibroblasts

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We recently demonstrated that diacylglycerol induced arachidonate release and prostaglandin E_2 synthesis in 3T3 fibroblasts, and greatly augmented prostaglandin E_2 synthesis in response to submaximal and maximal concentrations of bradykinin. We have now partially purified a phospholipase A_2 from the cells. When phosphatidyl[^{3}H]choline was used as substrate, several diacylglycerols augmented phospholipase A_2 activity. Diacylglycerol was effective at concentrations as low as 30 nM. Protein kinase C inhibition did not affect diacylglycerol's stimulation of phospholipase A_2. Diacylglycerol did not alter the calcium requirement for phospholipase A_2 or its pH optimum. The present study demonstrates that the effect of diacylglycerol to augment arachidonate metabolism is at the level of phospholipase A_2, itself.

Phospholipase A_2; Arachidonic acid; Diacylglycerol; Protein kinase C; (Fibroblast)

1. INTRODUCTION

We have shown that bradykinin stimulates phospholipase A_2 (PLA_2) activity in Swiss 3T3 fibroblasts to release arachidonate for prostaglandin E_2 (PGE_2) synthesis [1]. The primary substrate for the PLA_2 appeared to be phosphatidylcholine. We have also shown that in these cells diacylglycerols stimulate basal arachidonate release and PGE_2 synthesis, and that diacylglycerols markedly augment PGE_2 synthesis in response to bradykinin [2]. The site of diacylglycerol action was not defined, although protein kinase C (PKC) did not appear to be involved.

In the present study, we have used a partially purified membrane-bound PLA_2 from Swiss 3T3 fibroblasts to demonstrate that the effect of diacylglycerol is at the level of PLA_2.

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2. MATERIALS AND METHODS

2.1. *Purification of PLA_2*

Swiss 3T3 fibroblasts were maintained as previously described [1]. Cells were harvested by scraping from the plates in phosphate-buffered saline and pelleted at 50000 × *g* for 10 min. The pellet was resuspended in 25 mM Tris-Cl (pH 7.4), 1 mM EGTA, 1 mM deoxycholate and 1 M KCl, and sonicated with a probe sonicator 4 times for 5 s each, with 30 s between each burst, in an ice bath. The lysate was incubated at room temperature for 30 min, then centrifuged at 200000 × *g* for 60 min. The supernatant was diluted in 100 vols of the same buffer without KCl, concentrated to 10 ml by ultrafiltration through an Amicon PM10 membrane, then applied to a DEAE-cellulose (Whatman DE-52) column (1 × 10 cm) that had been equilibrated with 25 mM Tris-Cl (pH 7.4), 1 mM EGTA, and 1 mM deoxycholate. The column was eluted with 25 ml of the same buffer followed by a linear gradient of 0–500 mM NaCl in the same buffer. Fractions of 2 ml were collected and PLA_2 activity was assayed at pH 7 and 9. A single peak of PLA_2 activity (pH 9) was eluted at 100–200 mM NaCl. No PLA_2 was eluted that was more active at pH 7 than at pH 9. This partially purified enzyme was dialyzed against 20 mM Tris, pH 7, and used within 2 days.

2.2. *PLA_2 activity*

Phosphatidyl[^{3}H]choline was prepared by incubation of Swiss 3T3 fibroblasts with [^{3}H]choline for 30–48 h. Phospholipids were extracted [3] and the lipids were subjected

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to thin-layer chromatography [4]. The phosphatidylcholine fraction was scraped from the plate and was eluted with methanol, evaporated and stored in toluene/ethanol (50:50). Phosphatidylcholine concentration was estimated by measuring lipid phosphorus content [5].

For assay, a reaction mixture was prepared by adding labeled phosphatidylcholine to a 20 ml glass vial, with diacylglycerol where appropriate. The substrate was evaporated under N_2, then resuspended in 100 mM Tris, pH 9 (or other pH in selected experiments), containing 1 mM $CaCl_2$ and 1 mM deoxycholate to a final phosphatidylcholine concentration of 10 μM. The mixture was sonicated by probe 3 times for 5 s each, separated by 15 s intervals in an ice bath. For assay, 75 μl of this mixture was added to 12 × 75 mm glass tubes. The assay was begun by the addition of 25 μl of enzyme preparation and subsequent incubation at 37°C. The reaction was linear for at least 90 min. The reactions were terminated by the addition of 200 μl of methanol/chloroform/1 N HCl (2:1:0.01), followed by 100 μl of 2 M KCl. The phospholipids were separated by thin-layer chromatography [4], and lysophosphatidylcholine was scraped from the plate, eluted with methanol and radioactivity was quantitated by liquid scintillation spectroscopy.

2.3. *Materials*

All lipids were obtained from Sigma. Phorbol myristate acetate was obtained from Calbiochem. H-7 was obtained from Seikagaku America.

3. RESULTS

3.1. *Stimulation of partially purified phospholipase A_2 by diacylglycerols*

When oleoylacetylglycerol or dioleoylglycerol was present in the assay mixture, PLA_2 activity was markedly augmented (fig.1). A variety of diacylglycerols were able to stimulate PLA_2 activity (table 1). Among those tested, stearoylarachidonylglycerol has been shown to be a predominant diacylglycerol produced in cells [6]. Most of the diacylglycerols displayed similar effectiveness in stimulation of PLA_2. However, oleoylacetylglycerol was consistently less effective than longer chain diacylglycerols (fig.1 and table 1).

The concentration-response for stimulation of PLA_2 by dioleoylglycerol is shown in fig.2. At a concentration as low as 0.03 μM, dioleoylglycerol significantly stimulated PLA_2 activity. Other diacylglycerols displayed similar concentration-response patterns (not shown).

3.2. *Role of protein kinase C in stimulation of PLA_2 by diacylglycerols*

In our previous study [2], we demonstrated that diacylglycerols markedly augment arachidonate metabolism in Swiss 3T3 cells. While diacylglycerols activated PKC in the cells, we excluded this enzyme from playing a major role in stimulation of arachidonate metabolism, since neither inhibition nor down-regulation of PKC blocked diacylglycerols' effects. In our partially purified PLA_2 preparation, under the conditions of the PLA_2 assay, we detected no apparent PKC activity (table 2). However, if assay conditions were altered to be optimal for PKC, we did find evidence for protein kinase activity in the preparation (table 2). Thus, we tested the ability of the PKC inhibitor H-7 to affect diacylglycerol-stimulated PLA_2 activity. Over a wide concentration range, H-7 did not affect diacylglycerol-stimulated PLA_2 activity. In the presence of 100 μM H-7, 4835 ± 370 cpm lysoPC were formed, compared to 4886 ± 322 cpm in the presence of dioleoylglycerol alone (1420 ± 235 cpm in the absence of dioleoylglycerol). As a

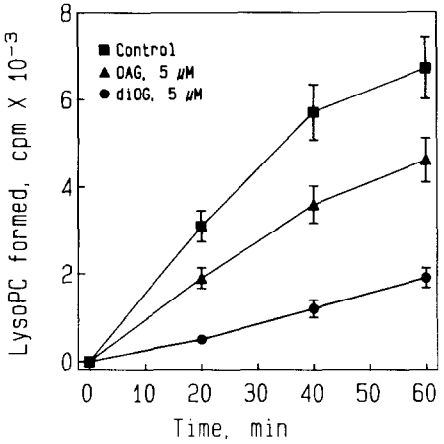

Fig.1. Oleoylacetylglycerol (OAG) and dioleoylglycerol (diOG) stimulate PLA_2 activity. Data are triplicate tubes assayed at pH 9 from 1 of 3 similar experiments.

Table 1

Comparison of the ability of several diacylglycerols to stimulate PLA_2

	LysoPC (cpm)
Control	862 ± 94
Oleoylacetylglycerol, 5 μM	2654 ± 285
Dioleoylglycerol, 5 μM	3562 ± 430
Dipalmitoylglycerol, 5 μM	3238 ± 264
Stearoylarachidonylglycerol, 5 μM	3764 ± 386

Incubations were at pH 9 for 60 min at 37°C. Data are from 1 of 2 similar experiments

Table 2

Protein kinase C activity in the partially purified PLA_2 preparation

	cpm ^{32}P into histone
Control	24 ± 20
OAG, 5 μM	30 ± 14
PKC assay buffer + OAG	96 ± 24
Initial homogenate + OAG	370 ± 26

Mean ± SE of triplicate determinations in 1 of 2 similar experiments. Incorporation of ^{32}P from [γ-^{32}P]ATP into histone (type III-S) was assayed in the PLA_2 assay buffer plus radioactive ATP or in PKC assay buffer (400 μg/ml phosphatidylserine, 1 mM $CaCl_2$, and 5 μM oleoylacetylglycerol (OAG) for 2 min) [14]. Homogenates of 3T3 cells were also assayed in PKC assay buffer and adjusted to the same amount of protein added (10 μg) as the partially purified PLA_2 preparation

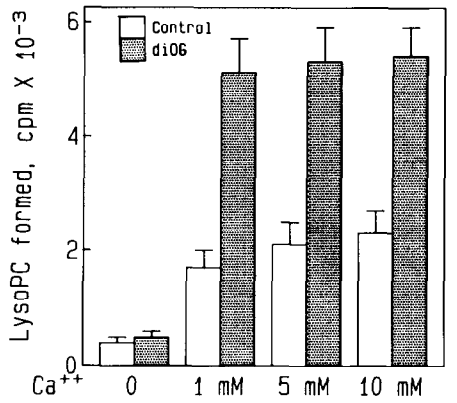

Fig.3. Effect of calcium on the ability of dioleoylglycerol (diOG) to stimulate PLA_2. The ordinate is the amount of calcium added to the assay buffer. Incubations were for 60 min at 37°C. Data are from 1 of 2 similar experiments.

final test of whether PKC might mediate diacylglycerols' ability to stimulate PLA_2, we prepared membranes from Swiss 3T3 cells that had been pretreated with 1 μM phorbol myristate acetate for 48 h, a treatment that completely down-regulates PKC [2]. Down-regulation of PKC had no effect on the ability of diacylglycerols to stimulate PLA_2 activity, since after down-regulation 3260 ± 386 cpm lysoPC was formed, compared to 3654 ± 270 in the presence of dioleoylglycerol alone (control 1128 ± 145 cpm).

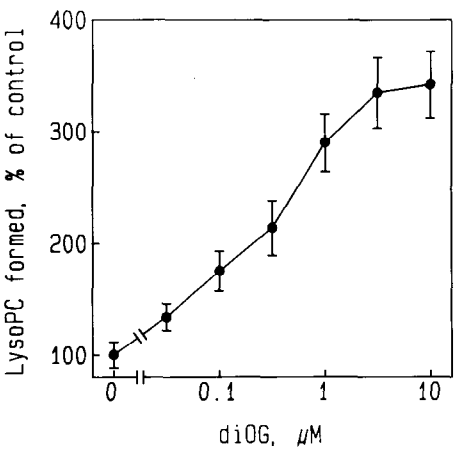

Fig.2. Concentration-response for stimulation of PLA_2 by dioleoylglycerol (diOG). Incubations were for 60 min at 37°C. Data are from 1 of 3 similar experiments. At 0.03 μM, dioleoylglycerol significantly stimulated PLA_2 ($p < 0.05$) when all 3 experiments were compared by Student's t-test for paired observations.

3.3. *Role of calcium in the ability of diacylglycerol to stimulate PLA_2*

Since most PLA_2s require calcium for activity, we tested the effects of altered calcium concentration on diacylglycerol-stimulated PLA_2 activity. In the absence of added calcium, diacylglycerol did not stimulate PLA_2 activity (fig.3).

4. DISCUSSION

The present study extends our previous observation that diacylglycerol stimulates arachidonate metabolism in Swiss 3T3 cells. We have now shown that diacylglycerol acts at the level of PLA_2 itself, markedly stimulating activity of a PLA_2 partially purified from Swiss 3T3 cells.

A variety of diacylglycerols were able to stimulate PLA_2 activity. However, oleoylacetylglycerol was consistently less effective than longer-chain diacylglycerols. It has been previously shown that diacylglycerols cause restructuring of phospholipid vesicles [7]. This may allow PLA_2 more ready access to its substrate.

It is particularly noteworthy that stearoylarachidonoylglycerol, a diacylglycerol that is formed in response to activation of a number of receptors [6], stimulated PLA_2 activity. We have no information about the mass of diacylglycerol formed in

Swiss 3T3 cells in response to agonists. In platelets, however, diacylglycerol (4–11 nmol/10^9 platelets) is generated in response to receptor activation [8], amounting to perhaps 1–2 mol% of total phospholipid. In our experiments, 0.3 mol% diacylglycerol significantly stimulated PLA_2. Thus, formation of diacylglycerol in response to receptor activation may be expected to stimulate PLA_2.

In our previous study utilizing intact Swiss 3T3 cells, diacylglycerol stimulation of arachidonate metabolism appeared to be independent of PKC. Under the PLA_2 assay conditions of the present study, PKC activity could not be detected. Further evidence against participation of PKC were the findings that neither PKC inhibition nor down-regulation affected diacylglycerol stimulation of PLA_2 activity. Also, experiments showed that in the absence of added calcium, PLA_2 was not activated. In the absence of added calcium, free calcium in the buffer was likely several micromolar [9]. This concentration would be adequate to fully activate PKC in the presence of diacylglycerol.

The mechanism by which diacylglycerol stimulated PLA_2 activity is unclear. Diacylglycerol did not affect the calcium requirement of the enzyme nor its pH optimum. The mechanism may relate to the ability of diacylglycerol to alter the structural organization of the substrate. It has been suggested that PLA_2 and PKC may contain similar regulatory sequences [10]. Thus, similar to its effect on PKC [11], diacylglycerol may directly bind to PLA_2 and activate it. Testing this possibility awaits further purification of PLA_2.

Similar ability of diacylglycerol to stimulate phospholipases has been observed for purified phosphatidylinositol-specific phospholipase C [12], and other PLA_2s [13]. However, this study correlated diacylglycerol activation of PLA_2 to a biological effect, potentiation of PGE_2 synthesis in response to receptor agonists [2]. This action of diacylglycerol may be physiologically relevant in that diacylglycerol, newly synthesized in response to a variety of agonists may activate PLA_2, leading to arachidonate release and metabolism, or other biochemical events mediated by PLA_2 metabolites.

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