The diacylglycerols dioctanoylglycerol and oleoylacetyl-glycerol enhance prostaglandin synthesis by inhibition of the lysophosphatide acyltransferase

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Prostanoids are synthesized by resident macrophages upon stimulation with diacylglycerols. Oleoylacetyl-glycerol and dioctanoylglycerol induced prostaglandin E and thromboxane synthesis in a time- and concentration-dependent manner. Both diacylglycerols inhibited the lysophosphatide acyltransferase, which is the key enzyme in the reacylation of arachidonic acid. By this mechanism the pool of free arachidonic acid available for prostanoid synthesis is increased. Both diacylglycerols were able to inhibit the membrane-bound lysophosphatide acyltransferase by a direct interaction independent of protein kinase C. Thus lysophosphatide acyltransferase could be shown to be a new target of these diacylglycerols, known as activators of protein kinase C.

INTRODUCTION

Prostanoid synthesis may be induced in macrophages by a great variety of physiological and pharmacological stimuli. In general, the increased rate of synthesis is not due to an activation of the cyclo-oxygenase or one of the subsequent enzymes but is dependent on an enhanced amount of arachidonic acid available as substrate [1]. Arachidonic acid is liberated from membrane phospholipids by phospholipases [2]. Thus activation of phospholipase A₂ leads to an increase in the pool of free arachidonic acid. The pool available for prostanoid synthesis, however, is in addition regulated by the reacylation of the fatty acid into membrane lipids. In macrophages it was shown that a considerable amount of liberated arachidonic acid was only measurable when the reacylating enzyme, lysophosphatide acyltransferase, was inhibited [3]. Inhibition of the acyltransferase by thiol compounds led to a large increase in prostanoid secretion [4]. This relation between enhanced synthesis of prostaglandins and inhibition of acyltransferase was also demonstrated in other cell types [5,6].

Among the lipidic compounds which induce arachidonic acid release and prostanoid synthesis, there are the short chain diacylglycerols diC₈ and OAG [7–10]. These compounds are known as activators of protein kinase C in assays *in vitro* [11]. The interaction between protein kinase C and the diacylglycerols is thought to take place in the plasma membrane [12]. It may be assumed that these lipids, when incorporated into the plasma membrane, might also influence other membrane-bound enzymes. In recent reports, protein kinase C-independent effects of OAG were described in neutrophils [13] and HL 60 granulocytes [14], but the molecular basis of the OAG action was not yet defined.

The lysophosphatide acyltransferase is a membrane-

bound enzyme and sensitive to changes in the surrounding lipids [15,16]. Thus it might also be influenced by intercalating diacylglycerols such as diC₈ and OAG. The enhanced prostanoid synthesis might then at least partially be caused by an impaired reacylation.

EXPERIMENTAL

Stimulation of macrophages with OAG and diC₈

Resident macrophages were obtained from the peritoneal cavity of DBA/2 mice as described recently [17]. They were preincubated for adherence for 2–3 h. After vigorous washing the cells were incubated for the times indicated with various concentrations of OAG or diC₈ (Sigma). The diacylglycerols were used after evaporation of the solvent chloroform/methanol (2:1, v/v) and brief sonication in the incubation medium (Dulbecco's modified Eagle medium; GIBCO Biocult).

Determination of prostaglandin E

The amount of prostaglandin E in the culture supernatant was determined by radioimmunoassay with specific antisera using a modified double-antibody method (V. Kaever, M. Goppelt-Strübe & K. Resch, unpublished work). The assay was highly sensitive, with a detection limit for prostaglandin E of 20 pg/ml. OAG and diC₈ did not interfere with the radioimmunoassay measurements.

Assay of LAT activity in macrophages

After removal of the supernatant, diacylglycerol-treated macrophages were homogenized by sonication in the assay buffer [20 mM-Hepes (pH 7.4)/0.14 M-KCl]. Protein was determined by the method of Bradford [18] with bovine serum albumin as standard. LAT activity

Abbreviations used: diC₈, 1,2-dioctanoyl-*sn*-glycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; LAT, lysophosphatide acyltransferase.

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was determined with [^{14}C]lysophosphatidylcholine and arachidonoyl-CoA as substrates [19]. Protein (8–20 μg) was incubated with 25 nmol of lysophosphatidylcholine, 10 nCi of 1-[^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine (Amersham International) and 10 nmol of arachidonoyl-CoA in a total volume of 0.5 ml of assay buffer. After incubation for 10 min at 37 °C, the reaction was stopped with methanol. The phospholipids were extracted, separated by t.l.c. and identified by comparison with authentic standards. The enzymic activity was calculated from the conversion of labelled precursor into phosphatidylcholine.

To determine the direct influence of OAG and diC_8 on the enzyme activity, homogenates of untreated macrophages were preincubated with the diacylglycerols for 5 min prior to the addition of lysophosphatidylcholine.

Preparation of plasma membranes

Highly purified plasma membranes were prepared from T-lymphocytes from calf as described in detail elsewhere [15]. The cells were broken by nitrogen cavitation and the plasma membranes purified by differential centrifugation.

Assay of LAT activity in plasma membranes

LAT activity was determined by an optical assay, based on the reaction of liberated coenzyme A with the dye 5,5'-dithiobis-(2-nitrobenzoic acid) [20]. After 5 min of preincubation of the plasma membranes (about 50 μg of protein/ml) with OAG or diC_8 , the reagent (Sigma) and arachidonoyl-CoA (20 μM) were added and the reaction mixture warmed to 37 °C for 5 min in the thermostat of the photometer. The reaction was started by the addition of lysophosphatidylcholine (50 μM). The specific activity was calculated from the initial rate of reaction using a molar absorption coefficient of $13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$. In control experiments it could be shown that the plasma membranes contained no activity of an arachidonoyl-CoA hydrolase.

RESULTS

Stimulation of prostaglandin synthesis

When resting macrophages derived from the peritoneal cavity of mice were incubated with OAG or diC_8 , the release of prostaglandins was enhanced. The increase was time- and concentration-dependent. As an example, the time course of prostaglandin E synthesis is shown in Fig. 1. Similar results were obtained for thromboxane, with the exception that the absolute amounts were lower.

Inhibition of LAT activity

The activity of the LAT was determined in homogenates of macrophages that had been incubated with OAG or diC_8 . Both diacylglycerols induced a concentration-dependent decrease in LAT activity (Fig. 2). The concentrations used were in the same range as those necessary for the induction of prostaglandin synthesis. When the diacylglycerols were added to homogenates of untreated cells immediately before performing the assay for LAT activity, they also inhibited the enzymic activity (Fig. 3).

Fig. 1. Increase in prostaglandin E synthesis by diC_8 and OAG

Resident peritoneal macrophages were incubated with diC_8 (25 $\mu\text{g/ml}$) (Δ), OAG (50 $\mu\text{g/ml}$) ($\square$) and without diacylglycerol ($\circ$) at a cell density of 5×10^5 cells/ml for the times indicated. The prostaglandin E content in the incubation medium was determined by radioimmunoassay. Bars indicate deviations between two experimental samples.

Fig. 2. LAT activity of resident peritoneal macrophages

Macrophages were incubated without ($\circ$) and with various concentrations of diC_8 (Δ) and OAG ($\square$) for 4 h. Then the activity of the LAT was determined in the homogenates. LAT activity of untreated macrophages was considered as 100%. Data shown are from a representative experiment out of a series of four similar ones.

Mechanism of the LAT inhibition

Further experiments were performed with an enzyme preparation enriched in highly purified plasma membranes from resting calf thymocytes. Both diacylglycerols inhibited the enzymic activity of the LAT; 50% inhibition was observed with concentrations in the same order as those of the substrates lysophosphatidylcholine and arachidonoyl-CoA (Table 1).

The optical assay was used for these studies to determine the initial rate of reaction at low concentrations of lysophosphatidylcholine, when the reaction was linear only for a few minutes. The enzymic reaction followed Michaelis-Menten kinetics with respect to the substrate lysophosphatidylcholine (Fig. 4). When the diacyl-

Fig. 3. Effect of diC₈ and OAG on LAT activity in cell homogenates

Untreated macrophages were homogenized and protein was determined. Samples (10 µg of protein) were incubated for 5 min without and with various concentrations of diC₈ and OAG before assaying the LAT activity. The activity of one sample without DAG was considered as 100%. Bars indicate deviations between two experimental samples.

Table 1. Inhibition of lysophosphatide acyltransferase by diC₈ and OAG

The activity of the lysophosphatide acyltransferase was determined in plasma membranes derived from T-lymphocytes as described in the Experimental section. Substrate concentrations were 50 nmol of lysophosphatidylcholine/ml and 20 nmol of arachidonoyl-CoA/ml. Data shown are means ± S.D. for three preparations.

	Concentration (nmol/ml) required for inhibition by:	
	25 %	50 %
diC ₈	36.8 ± 7.9	82.9 ± 14.0
OAG	27.0 ± 8.1	70.5 ± 15.0

Fig. 4. Kinetics of the interaction between LAT and diC₈ or OAG

LAT activity was measured in highly purified plasma membranes derived from T-lymphocytes as described in the Experimental section. Initial rates were determined in the absence (●) and presence of diC₈ (▲, a) and OAG (■, b). Data shown are from a representative experiment.

glycerols were used in low concentrations, the curves could formally be fitted by Michaelis-Menten kinetics, V_{max} , and the apparent K_m being enhanced (Figs. 4a and 4b). At higher concentrations of diC₈ or OAG the curves became irregular. These findings indicated that the diacylglycerols did not compete at the catalytic centre of the enzyme.

In the assay system, plasma membrane vesicles were incubated with lysophosphatidylcholine, which lyses the membranes at prolonged incubation or higher concentrations. In order to determine whether the intact membrane structure was necessary for the inhibitory effect of the diacylglycerols, the following experiments were performed (Fig. 5): the reaction was started with more than saturating concentrations of lysophosphatidylcholine, 70 and 200 µM. The enzymic activity of the LAT was the same at both concentrations and the reaction was inhibited by diC₈ in a concentration-dependent manner. When, however, the plasma membranes were preincubated with lysophosphatidylcholine and the reaction started with the second substrate, arachidonoyl-CoA, a different result was obtained: the enzymic activity of the partially solubilized enzyme was

Fig. 5. Influence of membrane integrity on the inhibition by diacylglycerols

Plasma membranes were incubated with various concentrations of diC₈. The enzymic reaction was started by the addition of lysophosphatidylcholine (■, 70 μM; ●, 200 μM) as described in the Experimental section (lower lines). The upper lines were obtained when the membranes were preincubated with lysophosphatidylcholine and the reaction started by the addition of arachidonoyl-CoA.

the same as the activity of the membrane-bound enzyme; the inhibition by diC₈, however, was greatly diminished.

DISCUSSION

The rate of an increased prostaglandin synthesis is regulated primarily by the availability of free arachidonic acid liberated from membrane phospholipids. Besides activation of phospholipases, inhibition of the reacylation could be shown to be an effective mechanism to enhance the pool of free arachidonic acid [3,4]. Substances used as inhibitors of the reacylating enzyme LAT were mercury compounds, e.g. thimerosal. However, LAT may also be inhibited by substrate analogues such as ether phospholipids, e.g. platelet activating factor [21]. We could now demonstrate that two diacylglycerols, OAG and diC₈, also inhibited the LAT.

When macrophages were treated with the diacylglycerols, the increase in prostaglandin synthesis correlated with a decrease in the activity of the lysophosphatide acyltransferase, measured in the homogenates of these cells. As diC₈ and OAG are activators of protein kinase C it seemed likely that the inhibition was mediated by the kinase. The inhibition of the LAT could also be achieved when the diacylglycerols were added to homogenates from control cells. Though no exogenous cofactors for protein kinase C were added, the homogenate might have provided sufficient ATP, Ca²⁺ and lipids to enable phosphorylation. Therefore, it was not clear whether the inhibition of the enzyme was mediated by protein kinase C or was due to a more direct interaction between the diacylglycerols and the LAT.

It was thus necessary to work with a pure membrane fraction under conditions where protein kinase C activity was excluded. These studies could not be undertaken with plasma membranes from peritoneal macrophages from mouse, because of the low number of macrophages

that can be obtained from one mouse and the amount of protein necessary to perform the enzymic assays. Plasma membranes from calf thymocytes were chosen for the following reasons: (i) the purity of the plasma membranes obtained by nitrogen cavitation and differential centrifugation is well documented [15,22]; (ii) several hundred micrograms of plasma membranes can be easily prepared; (iii) the specific activity of the LAT is higher in the plasma membrane than in other cellular membranes of T-lymphocytes; (iv) up to now, there is no evidence that the LAT shows qualitatively different characteristics in T-lymphocytes and macrophages. Protein kinase C activity could be excluded in the assay system used: none of the obligatory cofactors (Ca²⁺, phosphatidylserine and ATP) was added to the system, the plasma membranes were free of ATP and the inhibition could also be measured in the presence of EGTA (5 mM). Thus the inhibition of the LAT measured under these conditions was attributed to a direct interaction between the diacylglycerols and the LAT, independent of protein kinase C.

The catalytic activity of the LAT was the same when the enzyme was embedded in the plasma membrane or when the enzyme was partially solubilized by one of the substrates, lysophosphatidylcholine. The inhibition by the diacylglycerols, however, was dependent on the intact membrane and nearly abolished when the enzyme was solubilized by preincubation with lysophosphatidylcholine. Thus the fitting to the Michaelis-Menten curve, which suggested an increase in V_{max} , was due to the fact that at higher concentrations of lysophosphatidylcholine the membrane became partially solubilized. Instead of a competitive inhibition the diacylglycerols seem to act effectively only when the enzyme is constrained in the membrane.

The aspect of an inhibition of the reacylation as one mechanism to induce an increase in liberated arachidonic acid and prostaglandin synthesis has not yet been discussed in connexion with respective observations in macrophages [10] or in platelets, where OAG was shown to be a costimulator with ionophore without increasing the pool of arachidonic acid on its own [9].

The direct inhibition of the LAT is probably not the only mechanism by which the enhanced prostaglandin synthesis is induced by diC₈ and OAG. An additional protein kinase C-mediated impairment of the LAT is not excluded by the experiments shown above. Furthermore, there is evidence that phospholipases are also activated in cells stimulated with short chain diacylglycerols.

The expert technical assistance of Mrs. M. Golombek is gratefully acknowledged. This work was supported by grant GO 413/1-2 from the Deutsche Forschungsgemeinschaft and by grant 2091-B VII 3 Stiftung Volkswagenwerk, Land Niedersachsen.

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Received 13 May 1987/10 August 1987; accepted 21 August 1987