Diacylglycerol induces deacylation of phosphatidylinositol and mobilization of arachidonic acid in mouse macrophages

Comparison with induction by phorbol diester

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1,2-Dioctanoyl-*sn*-glycerol (2–50 μ M) was found, like phorbol myristate acetate (≥ 3 nM) to stimulate phospholipase A-type cleavage of phosphatidylinositol and the release of arachidonic acid from macrophage phospholipids. The 1,3 isomer of dioctanoylglycerol was inactive, whereas racemic 1,2-dioctanoylglycerol was half as potent as the 1,2-*sn* enantiomer. Dioctanoylglycerol-induced deacylation of phosphatidylinositol was only partly dependent on extracellular calcium but was more severely inhibited by depletion of intracellular calcium. Chlorpromazine inhibited the deacylation of phosphatidylinositol, whereas inhibitors of cyclo-oxygenase and lipoxygenase were ineffective. Since both phorbol myristate acetate and 1,2-dioctanoyl-*sn*-glycerol are known to activate protein kinase C, the results suggest that this kinase is involved in the sequence of events leading to release of arachidonic acid in macrophages.

INTRODUCTION

Much attention has recently been focused on the physiological role of phosphoinositide turnover and its possible relationship to intracellular mobilization of Ca^{2+} and the activation of a Ca^{2+} - and phospholipid-dependent protein kinase C. Diacylglycerol increases the affinity of kinase C for Ca^{2+} and certain phorbol diesters are even more potent in this respect, probably because of a slower rate of elimination (for reviews see [1–3]).

We have recently found that PMA selectively enhances the degradation of PtdIns by a phospholipase A pathway with a concomitant release of arachidonic acid from both PtdIns and other phospholipids in mouse peritoneal macrophages [4]. It was therefore of interest to investigate the effects of exogenously added diacylglycerol, since this lipid intermediate is considered to be the physiologically active messenger.

The results show that 1,2-dioctanoyl-*sn*-glycerol, previously shown to act on intact cells [5–7], causes both activation of phospholipase A, as judged by the accumulation of lysoPtdIns and GroPIns, and release of arachidonic acid. It is therefore likely that activation of protein kinase C plays a critical role in the signal pathway leading to phospholipase A activation and mobilization of arachidonic acid in mouse macrophages.

EXPERIMENTAL PROCEDURES

Preparation, labelling and stimulation of macrophages

Resident peritoneal cells were harvested in 4 ml of Medium 199 (M 199; Flow Laboratories) containing 1% heat-inactivated fetal calf serum and heparin (20 units/ml) from outbred female albino mice (Antimex, Stockholm, Sweden) by a modification of the procedure

described by Cohn & Benson [8]. The cells were plated (approx. 4×10^6 cells/35-mm well) onto plastic six-well Linbro tissue culture dishes and incubated in an atmosphere of 5% CO_2 in air. Non-adherent cells were removed 2–3 h after plating and to each dish was then added 1 ml of M199 (Earle's salts supplemented with 10 mM-Hepes) containing 10% fetal calf serum. The cells were labelled for 24 h with 2 μ Ci of [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (Amersham; sp. radioactivity 100–135 Ci/mmol) or 10 μ Ci of *myo*-[2- ^3H]inositol (Amersham; sp. radioactivity 15 Ci/mmol). Thereafter the cells were washed several times with phosphate-buffered saline and were then allowed to equilibrate for $\frac{1}{2}$ –1 h in fresh serum-free M199 before the start of the experiment. The experiments were conducted in a medium where one-third of M199 was replaced by Earle's balanced salt solution, containing 150 mM-LiCl instead of NaCl, to minimize degradation of InsP formed [9]. When the concentration of Ca^{2+} was varied, Earle's balanced salt solution containing 50 mM-LiCl was used. The 1,2-*sn*-DOG was prepared by treatment of the parent phosphatidylcholine (Serva) with *Bacillus cereus* phospholipase C_1 [10] essentially as described by Ebeling *et al.* [6]. An equilibrium mixture of 1,2-*rac*-DOG and 1,3-DOG was a generous gift from Dr. Lennart Krabisch of this department. 1,2-*sn*-DOG, 1,2-*rac*-DOG and 1,3-DOG were purified by h.p.l.c. on a 250 mm $\times$ 4 mm column containing LiChrosorb Si60 (5 μ m, Merck) using the solvent hexane/propan-2-ol (19:1, v/v) at a flow rate of 1 ml/min. The concentration of DOG was then determined after alkaline hydrolysis by a kit for the enzymic determination of glycerol (Boehringer Mannheim). Phorbol diesters (Sigma) or DOG as well as A23187 (Boehringer), chlorpromazine, indomethacin and esculetin (Sigma) were added in 5–10 μ l of dimethyl sulphoxide. To control dishes was added the same amount of dimethyl sulphoxide.

Abbreviations used: GroPIns, glycerophosphoinositol; InsP, inositol phosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; DOG, dioctanoylglycerol; α -PDD, 4 α -phorbol 12,13-didecanoate; β -PDD, 4 β -phorbol 12,13-di-decanoate; PMA, 4 β -phorbol 12-myristate 13-acetate.

Extraction and analysis

The culture medium was removed and the cells were scraped off the dish in 1 ml of ice-cold 50 mM-HCl (or 10 mM-HCl when cells were labelled with [3 H]arachidonic acid) and lipids were extracted with 6 ml of chloroform/methanol (1:1, v/v) containing 0.05% 2,6-di-*t*-butyl-*p*-cresol as an antioxidant. Phase separation was effected by centrifugation after addition of 2 ml of 50 or 10 mM-HCl. Lipid standards were added to the extract as carriers and to aid in identification of the lipids. The lower phase was withdrawn, taken to dryness under N_2 and the extracted lipids were then dissolved in chloroform/methanol (2:1, v/v).

Lipids labelled with [3 H]arachidonic acid were separated on precoated thin-layer plates (silica gel 60; Merck) developed in chloroform/methanol/acetic acid/water (25:20:3:0.3, by vol. [11]). Phospholipids labelled with [3 H]inositol were separated on silica gel H (Merck) plates impregnated with 1% potassium oxalate, using the solvent system chloroform/methanol/28% ammonia/water (45:45:2.5:9, v/v [12]). Lipids were visualized with I_2 vapour, and appropriate bands were scraped into scintillation vials containing 1 ml of methanol/water (1:1, v/v). After addition of 10 ml of ES 299 (Packard) the radioactivity was determined in a Packard model 4530 scintillation spectrometer.

Water-soluble inositol compounds in the culture medium and the polar phase of the cell extract were analysed on small polypropylene columns containing 1 ml of AG 1-X8 in formate form (200–400 mesh, Bio-Rad), by a stepwise gradient of ammonium formate as previously described [4,13].

Data shown in Figs. 1–7 are representative of at least two experiments in each case.

RESULTS AND DISCUSSION

PMA was previously shown to enhance the deacylation of PtdIns in mouse peritoneal macrophages [4]. As shown in Fig. 1, degradation of PtdIns prelabelled with

Fig. 1. Dose-response for PMA-induced degradation of PtdIns

Peritoneal macrophages were labelled for 24 h with [3 H]inositol and then stimulated with various concentrations of PMA for 15 min. (a) PtdIns; (b) lysoPtdIns ($\blacktriangle$), GroPIns ($\bullet$) and InsP ($\blacksquare$).

Fig. 2. Time course of PMA-induced degradation of PtdIns

Prelabelled macrophages were stimulated with PMA (8 nM) for the time indicated. (a) PtdIns, (b) InsP, (c) lysoPtdIns, (d) GroPIns.

Table 1. Comparison between the effects of different phorbol diesters on phospholipid degradation

Mouse macrophages were prelabelled for 24 h with either [^3H]arachidonic acid or [^3H]inositol and were then exposed to $0.8\ \mu\text{M}$ -PMA, β -PDD or α -PDD for 15 min. The results show the release of radioactivity from cellular phospholipids, expressed as a percentage of total recovered activity, and are the mean $\pm$ S.E.M. for three experiments. On average, each culture contained 1.3×10^6 d.p.m. and 1.3×10^6 d.p.m. of ^3H after labelling with arachidonic acid and inositol, respectively.

	Change from control (%)	
	[^3H]Arachidonic acid	[^3H]Inositol
PMA	$+6.3 \pm 1.2$	$+7.3 \pm 1.2$
β -PDD	$+5.0 \pm 1.7$	$+5.2 \pm 0.9$
α -PDD	-0.3 ± 0.4	-0.2 ± 0.3

[^3H]inositol was observed already at $3\ \text{nM}$ -PMA when the cells were exposed to the compound for 15 min. At this concentration only phospholipase(s) A products, i.e. lysoPtdIns and GroPIns, accumulated. At higher concentrations of PMA there was also formation of InsP.

Fig. 3. Mobilization of phospholipid-bound arachidonic acid in response to PMA

Peritoneal macrophages were labelled for 24 h with [^3H]arachidonic acid and then stimulated with various concentrations of PMA for 15 min. (a) PtdEtn (●) and PtdCho (▲), (b) PtdIns.

Fig. 4. Dose-response for DOG-induced degradation of PtdIns

Peritoneal macrophages were labelled for 24 h with [^3H]inositol and then stimulated with various concentrations of 1,2-*sn*-DOG for 15 min. (a) PtdIns; (b) lysoPtdIns (▲), GroPIns (●) and InsP (■).

The time course of PMA-induced PtdIns degradation (Fig. 2) was such that the phospholipase A products lysoPtdIns and GroPIns predominated early in the time course while the formation of InsP occurred only later. It is therefore possible that the InsP which accumulated was formed by cleavage of GroPIns rather than by phospholipase C attack on phosphoinositides, especially since there were no significant changes in the labelling of inositol bis- or tris-phosphate [4].

As shown in Fig. 3, PMA also induced release of phospholipid-bound arachidonic acid. The concentration-dependence for PMA-induced degradation of arachidonic acid-labelled phospholipids was similar to that for the deacylation of inositol-labelled PtdIns. The arachidonic acid mobilized from PtdIns, PtdEtn and PtdCho was largely released from the cells as oxygenated metabolites.

If the effects of PMA described above depend on a specific interaction of PMA with a phorbol ester

Fig. 5. Mobilization of phospholipid-bound arachidonic acid in response to DOG

Peritoneal macrophages were labelled for 24 h with [^3H]arachidonic acid and then stimulated with various concentrations of 1,2-*sn*-DOG for 15 min. (a) PtdEtn (●) and PtdCho (▲); (b) PtdIns.

receptor, such as protein kinase C, one would expect only the 4β stereoisomer of phorbol diester to be active [14]. Table 1 shows the effects of the 4α - and 4β -isomers of PDD, compared with the effects of PMA. Clearly, the 4α -isomer induced neither deacylation of PtdIns nor mobilization of arachidonic acid, while the β -isomer was almost as effective as PMA at $0.8 \mu\text{M}$ concentration.

We then investigated whether an exogenously added diacylglycerol would have effects similar to those of the phorbol diesters. Indeed, in the concentration range earlier found to cause activation of protein kinase C in intact cells [5–7], 1,2-*sn*-DOG caused an extensive deacylation of PtdIns (Fig. 4) as well as release of arachidonic acid from PtdIns, PtdEtn and PtdCho (Fig. 5). Interestingly, the positional isomer 1,3-DOG

Fig. 6. Positional specificity of DOG-induced phospholipid degradation

Mouse macrophages prelabelled for 24 h with either [^3H]arachidonic acid (a) or [^3H]inositol (b) were exposed to various concentrations of DOG for 15 min. Change from control denotes the release of radioactivity from phospholipids in treated cultures compared with controls and is expressed as a percentage of total recovered activity. 1,2-*sn*-DOG (●), 1,2-*rac*-DOG (▲) and 1,3-DOG (■).

Table 2. Calcium-dependence of DOG-stimulated degradation of PtdIns

Peritoneal macrophages prelabelled with [^3H]inositol were incubated for 5 min prior to stimulation with DOG ($20 \mu\text{M}$, 15 min) at various concentrations of Ca^{2+} or in a Ca^{2+} -free medium containing EGTA (0.1 mM) or EGTA plus A23187 ($2 \mu\text{M}$). Values are given as a percentage of those obtained after addition of DOG in medium containing 1.8 mM-Ca^{2+} (the mean of two experiments).

	Radioactivity (% of control)	
	LysoPtdIns + GroPIns	InsP
0.2 mM- Ca^{2+}	87	71
EGTA	65	78
EGTA + A23187	38	3

Fig. 7. Time course of DOG-induced degradation of PtdIns

Prelabelled macrophages were stimulated with 1,2-*sn*-DOG (20 μ M) for the time indicated. (a) PtdIns, (b) InsP, (c) lysoPtdIns, (d) GroPIns.

Table 3. Effects of chlorpromazine and inhibitors of arachidonic acid oxidation on DOG-induced degradation of PtdIns

Peritoneal macrophages prelabelled with [3 H]inositol were incubated for 15 min with chlorpromazine (20 μ M) or indomethacin (2 μ M) and/or esculetin (10 μ M) prior to stimulation with DOG (20 μ M) for further 15 min. Values are given as a percentage of those obtained after addition of DOG alone (the mean of two experiments).

	Radioactivity (% of control)	
	LysoPtdIns + GroPIns	InsP
Chlorpromazine	59	23
Indomethacin	106	109
Esculetin	90	98
Indomethacin + esculetin	109	90

was inactive, while the racemic mixture of 1,2-*sn*- and 2,3-*sn*-DOG was approximately half as active as 1,2-*sn*-DOG (Fig. 6). This indicates that the 2,3-*sn*-form is either completely inactive or a very weak agonist/antagonist. The specificity reported here agrees well with that reported for the activation of protein kinase C *in vitro* [15].

The time course of 1,2-*sn*-DOG-induced degradation of PtdIns is shown in Fig. 7. Radiolabelled lysoPtdIns and GroPIns accumulated more rapidly than did InsP, as was the case when the cells were exposed to PMA. Thus, also here, InsP might have formed by further

cleavage of GroPIns. To assess further the contribution of the phospholipase A and C pathways to the observed increase in InsP, we studied the influence of Ca^{2+} depletion on the formation of phospholipase A products and InsP. Table 2 demonstrates that the accumulation of InsP was inhibited to an even larger extent by ionophore-aided depletion of intracellular Ca^{2+} , than that of immediate phospholipase A products. This supports the possibility that InsP was formed via GroPIns [16], since the zymosan-induced formation of InsP (most likely via phospholipase C) was unaffected by Ca^{2+} depletion, while the deacylation pathway was strongly inhibited [4]. As shown in Table 3, the 1,2-*sn*-DOG-induced formation of InsP was also inhibited to a large extent by chlorpromazine, again in contrast to what was earlier observed with zymosan [4]. Inhibitors of cyclo-oxygenase (indomethacin) and lipoxygenase (esculetin), on the other hand, either alone or in combination did not markedly influence DOG-stimulated PtdIns degradation (Table 3), indicating that the products of these pathways did not play a role in the activation process.

We have observed effects similar to those of DOG, although smaller, by other diacylglycerols such as 1-oleoyl-2-acetyl-glycerol and 1,2-didodecanoylglycerol. Treatment of the cells with exogenous phospholipase C from *Clostridium perfringens* (15–40 μ g), which preferentially degrades PtdCho but not at all PtdIns [17], was accompanied by increased formation of both diacylglycerol and phosphatidic acid, enhanced deacylation of PtdIns and release of arachidonic acid (results not shown). Under these conditions there were only minor changes in InsP. The addition of PtdIns-specific phosphodiesterase (5–10 μ g of phospholipase C_{II} from *B. cereus* [10]) caused 9% degradation of [3 H]inositol-

labelled PtdIns ($n = 3$) in 30 min with an accumulation of InsP, largely the cyclic form, in the culture medium. However, although this was accompanied by a 2–3-fold increase in arachidonic acid-labelled diacylglycerol there was no increase in phosphatidic acid and hardly any net release of arachidonic acid. This indicates that long-chain diacylglycerol generated in only limited amounts in the outer half of the plasma membrane was available neither to protein kinase C nor to diacylglycerol kinase.

PMA has previously been shown to activate phospholipid degradation in some cell types ([4] and references therein), while this compound and other protein kinase C activators (oleoylacetyl glycerol and didecanoyl glycerol) are ineffective in releasing arachidonic acid from platelet phospholipids [18–20]. However, these agents potentiate the effects of calcium ionophore in platelets [19,20]. The present work shows that diacylglycerol is a sufficient stimulus for release of arachidonic acid in intact mouse macrophages. This stimulation is, in addition, highly specific for the naturally occurring 1,2-*sn*-isomer of diacylglycerol. From available data on the volume and phospholipid content of macrophages [21,22] it can be calculated that a relatively small fraction of the phospholipid content of these cells needs to be degraded, via cell surface receptor activation, to achieve a cellular level of diacylglycerol comparable with that used in the present study.

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